POLO-LIKE KINASE INTERACTING PROTEINS IN FISSION YEAST

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Abstract

The cell cycle needs to be regulated accurately for new cells to receive the correct physiological components and genetic material. Polo-like kinases (Plks) are conserved proteins that perform multiple roles throughout the cell cycle. The *Schizosaccharomyces pombe* Plk, Plo1, is known to control bipolar spindle formation, exit from mitosis and cytokinesis. How one protein achieves such diverse functions is a major question addressed in this study. Interacting-proteins may mediate Plo1's regulatory functions. To find Plo1-interacting proteins, a yeast two-hybrid screen was performed using a meiotic cDNA library and full-length Plo1 as bait. Nine proteins were found, SPAC9.11, SPAC26H5.05, Sum2, Htb1, Ctt1, Zym1, Kms1, Kms2 and Sid4.

Plo1 localises to the spindle pole body (SPB) for part of the cell cycle, so the Plo1-interacting SPB proteins, Sid4, Kms1 and Kms2, were studied further. Sid4 is required for septation as it anchors all components of the septation pathway to the SPB, while Kms1 and Kms2 are important for integrity of the SPB. To find the biological significance of the interactions between Plo1 and these SPB proteins, attempts were made to disrupt the interaction by mutations. For this purpose, firstly regions responsible for the interaction were identified, and then mutations were made in the SPB proteins by random mutagenesis of this region.

For Sid4, I isolated two point mutations, which had greatly weakened interaction with Plo1. To study the effect of disrupting the interaction *in vivo*, the two *sid4* mutants were

expressed from a fission yeast promoter in the *sid4* temperature-sensitive mutant. Both point mutants of Sid4 that had weakened interaction with Plo1 were able to rescue the temperature-sensitve *sid* mutant with a similar strength as that of wild-type *sid4* under the same promoter.

The identification of potential Plo1 interacting proteins and mutants defective in these interactions will be an important step to understand cell cycle control by Plo1.

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List of Abbreviations

3-AT	3-aminotriazole
AD	Activation Domain
ADH1	Alcohol Dehydrogenase 1
Amp	Ampicillin
APC	Anaphase Promoting Complex
ATP	Adenosine 5'-triphosphate
BD	DNA Binding Domain
BLAST	Basic Local Alignment Search Tool
bp	Base pair
°C	Degrees Celsius
cDNA	Complementary Deoxyribonucleic Acid
cdc	Cell Division Cycle
CDE/CHR	Cell (cycle gene) Homology Region
Cdk	Cyclin-dependent Kinase
C-terminal	Carboxy-terminal (of a Protein)
cut	Cell Untimely Torn
dma	Defective in Mitotic Arrest
dNTP	Deoxynucleoside Triphosphate
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
g	Gram
G1	First Gap Phase in the Cell Cycle (Part of Interphase)
G2	Second Gap Phase in the Cell Cycle (Part of Interphase)
G2/M	Transition from Second Gap Phase to Mitosis
GAP	GTPase Activating Protein
GEF	Guanine Nucleotide Exchange Factor
HRP	Horseradish Peroxidase
kb	Kilobase
kD	KiloDalton
kms	Karyogamy and Meiotic Segregation
lacZ	Beta-galactosidase Gene
M	Molar
M-phase	Mitosis Phase of Cell Cycle
MAP	Microtubule-associated Protein
MEN	Mitotic Exit Network (of Genes)
mg	Milligram
ml	Millilitre
mM	Millimolar
MMS	Methylmethane Sulfonate
MPF	Maturation Promoting Factor
mRNA	Messenger Ribonucleic Acid
MTOC	Microtubule organising centre
N-terminal	Amino-terminal (of a Protein)

ng	Nanogram
nmt	No Message with Thiamine
nt	Nucleotide
ORF	Open Reading Frame
p	Plasmid
PAGE	Polyacrylamide Gel Electrophoresis
Pav	Pavarotti
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
Pfam	Protein Families (Database)
PKA	Protein Kinase A
Plks	Polo-like Kinases
Plk1	Human or Murine Polo-like Kinase
Plo1	Fission Yeast Polo-like Kinase
Plx	Xenopus Polo-like Kinase
pmol	Picomole
RNA	Ribonucleic acid
rpm	Revolutions Per Minute
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
S-Phase	(DNA) Synthesis Phase of Cell Cycle
SDS	Sodium Dodecyl Sulphate
sid	Septation Initiation Defective
SIN	Septation Initiation Network (of genes)
SPAC	*Schizosaccharomyces pombe* (A) First Chromosome
SPBC	*Schizosaccharomyces pombe* (B) Second Chromosome
SPCC	*Schizosaccharomyces pombe* (C) Third Chromosome
SPB	Spindle pole body
stf	Suppressor of (cdc) Twenty-Five
Sum	Suppresor of Uncontrolled Mitosis
TAE	Tris/Acetate Buffer
TBS	Tris-buffered Saline
TE	Tris-EDTA (buffer)
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
µg	Microgram
µl	Microlitre
UAS	Upstream Activating Sequence
V	Volts
v/v	Volume Per Volume
w/v	Weight Per Volume
X-galactose	5-bromo-4-chloro-3-indolyl-β-D-galactoside
xPlkk1	Xenopus Polo-like Kinase Kinase

Amino acid Abbreviations

Amino Acid	3-letter code	1-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutatmate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1. Introduction

1.1 General introduction to the cell cycle

Cells are the fundamental unit of life. They grow, replicate genetic material, segregate the replicated chromosomes and divide to form two new daughter cells. The cell division cycle is divided into four phases, growth, synthesis of DNA, a second phase of growth and then, mitosis (the division of genetic material) (Murray and Hunt, 1993).

Key proteins regulate the transitions between phases of the cell cycle. Cyclins and their partner kinases regulate cell cycle events. Cyclins are a group of proteins that are periodically degraded at the end of mitosis (Hunt, 2004). Cyclin-dependent kinases (Cdks) drive the cell division cycle and regulate the timing of various phases by the degree of their activity (Murray and Hunt, 1993). Cyclins are required for Cdk activity and their degradation leads to Cdk inactivation. This level of control ensures an accurate process so that each daughter cell receives the correct number of chromosomes. During mitosis, it is cyclin B and the activity of Cdk1 that drive the cell cycle. Together cyclin B and Cdk1 are referred to as maturation-promoting factor (MPF), for their ability to induce mitosis (Lohka et al., 1988). MPF is regulated by its localisation to the nucleus, activated by its dephosphorylation and shut off by degradation of one component, cyclin B, which is required for Cdk activity (Jackman et al., 2003; Toyoshima-Morimoto et al., 2001; Yuan

et al., 2002). Cdk1 phosphorylates its substrates and activates certain pathways or networks of genes to induce the changes that occur in preparation for the division of genetic material, such as duplication of microtubule organising centres, chromosome condensation, and spindle formation (Liu and Erikson, 2002; Miake-Lye and Kirschner, 1985; Newport and Kirschner, 1984). The spindle is the apparatus that separates chromosomes and is composed of microtubules. Cohesion between the sister chromatids is broken and then, cyclin B is degraded, whereupon the sister chromatids are pulled apart (Alexandru et al., 2001; Ciosk et al., 1998; Gruber et al., 2003; Shirayama et al., 1998). As MPF activity is decreased, the chromosomes decondense and the spindle is disassembled (Holloway et al., 1993).

In addition to cyclin-dependent kinases, polo-like kinases also control phases of the cell cycle (Glover et al., 1996). Plks affect Cdk activity, but Plks also depend on Cdks for their activity (Nigg et al., 1996; Ohi and Gould, 1999). Both ensure accurate timing of phases of the cell division cycle. Plks are essential proteins that are required for several events, including entry into mitosis, bipolar spindle formation, sister chromatid segregation, exit from mitosis and cytokinesis (Glover et al., 1996). Also in meiosis, polo-like kinases are responsible for the monopolar attachment of kinetochores of sister chromatids, so homologous chromosomes can be separated (Lee and Amon, 2001; Lee and Amon, 2003).

Polo-like kinases (Plks) are a family of conserved serine-threonine protein kinases, of which the founding member was identified in *Drosophila melanogaster* (Sunkel and Glover, 1988). Mutants of the *Drosophila* gene *polo* had a phenotype in which cells exhibited disorganised spindles with abnormal poles. The gene was called *polo*, which is the Spanish word for pole (Llamazares et al., 1991; Sunkel and Glover, 1988). In budding yeast, Cdc5 was found to be homologous in sequence to Polo, but *cdc5* mutants exhibited cytokinetic defects (Kitada et al., 1993), a very different phenotype from defective spindle formation in *Drosophila*. Studies in fission yeast showed that *plo1* mutants arrested in metaphase with abnormal spindles, monopolar spindles and overcondensed chromosomes, or underwent nuclear division without septation (Ohkura et al., 1995). Later studies in *Drosophila* also showed cytokinetic defects in *polo* mutants (Carmena et al., 1998). Thus, polo-like kinases are involved in bipolar spindle formation and cytokinesis.

How one kinase is able to perform such diverse roles is a major question. The hypothesis presented in this thesis is that Polo-like kinases interact with other proteins in order to exert their functions.

1.2. Structure of Polo-like kinases

Polo-like kinases are found in eukaryotes, but not in plants. They promote entry into mitosis by regulating the G2/M transition, encourage centrosome maturation, are

Table 1.1. The polo-like kinases found in different model organisms

Organism	Polo-like kinase	Unusual Polo-like kinase
S. pombe	Plo1 [1]	
S. cerevisiae	Cdc5 [2]	
D. melanogaster	Polo [3,4]	Sak [5]
C. elegans	Plk-1 [6] Plk-2 [7] Plk-3 [8]	
X. laevis	Plx1 [9] Plx2 [11] Plx3 [11]	Sak [10]
R. norvegicus	Plk1 [12] Snk [14] Cnk [15]	Sak [13]
M. musculus	Plk [16] Snk [18] Plk3 [20], Cnk [21]	Sak isoform a [17] Sak isoform b [19]
H. sapiens	Plk1 [22] Plk2, Snk [24] Plk3, Cnk/Fnk/Prk [25]	Sak [23]

The following are references for the proteins listed above or National Library of Medicine accession numbers for protein sequences. 1- (Ohkura et al., 1995), 2- (Kitada et al., 1993), 3- (Sunkel and Glover, 1988), 4- (Llamazares et al., 1991), 5- NP_649324, 6- NP_741243, 7-NP_491036, 8- S44841, 9- (Kumagai and Dunphy, 1996), 10- AAH60363, 11- (Duncan et al., 2001), 12- (Fan et al., 2003), 13- XP_227064, 14- NP_114009,
15- XP_342889, 16- NP035251, 17 –AAH57940, 18- AAH06880, 19- NP_775261, 20- AAH63051, 21- NM_013807, 22- AAP3645825, 23- AAH36023, 24- AAH13879 ,25- AAH13899

responsible for bipolar spindle formation, enhance sister chromatid segregation and they control exit from mitosis and initiation of cytokinesis. Table 1.1 shows the different types of polo-like kinases found in model organisms. Three Plks exist in vertebrates. Human Plk1 (Golsteyn et al., 1995), murine (Matsubara et al., 1995), rat Plk (Fan et al., 2003), and *Xenopus* Plx1 (Kumagai and Dunphy, 1996) are most like the originally identified Polo. Plk2 is also referred to as serum-inducible kinase, or Snk (Clay et al., 1993).

#242]. Plk3 was initially identified as an fibroblast growth factor-inducible kinase (Fnk) or cytokine-inducible kinase (Cnk), and has also been referred to as polo-related kinase (Prk) (Donohue et al., 1995). There is another related kinase in higher organisms (*Drosophila*, *C. elegans* and vertebrates) referred to as Sak. Sak is a stress-activated kinase only distantly related to the Plks (Fode et al., 1994; Hudson et al., 2001; Leung et al., 2002).

Alignments of the various polo-like kinases reveal two domains within the protein. At the amino-terminal end is the catalytic domain and at the carboxy-terminal end, the non-catalytic domain (Glover et al., 1996; Ohkura et al., 1995). The catalytic domain contains an invariant lysine and an arginine-glycine rich loop, which contribute to kinase activity (Figure 1.2). The non-catalytic domain consist of two polo boxes in Plks, consisting of conserved residues (Figure 1.1 and 1.3) that produce a recognisable structural fold (Elia et al., 2003a; Elia et al., 2003b). A linker region between the two domains exists, the last 45

amino acids of which is referred to as the polo cap (Elia et al., 2003b) (Figure 1.3). The polo cap is required for protein solubility in human Plk1 and may stabilise the structure of the non-catalytic domain. Sak is unique in that it only has one polo box in its non-catalytic domain (Hudson et al., 2001; Leung et al., 2002).

The non-catalytic domain is responsible for interactions with all known Plk interacting proteins, including substrates; although Plks kinase domain would interact with the phosphorylation site, it is the C-terminal polo box domain (PBD) that initially interacts prior to phosphorylation (Jang et al., 2002; Lee et al., 1999; Reynolds and Ohkura, 2003; Song et al., 2000). Polo boxes appear to mediate interaction with multiple proteins; some but not all of these proteins are substrates for the kinase domain of the molecule (Reynolds and Ohkura, 2003). Deletion or mutation in the non-catalytic domain diminishes binding (Jang et al., 2002; Reynolds and Ohkura, 2003; Song et al., 2000).

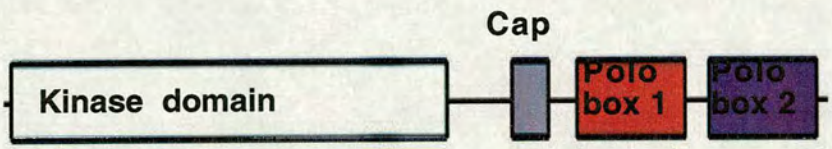

Figure 1.1. Schematic structure of Plk family protein. The white box represents the catalytic domain; the grey box, the polo cap; while the red box represents the first polo box, and the purple box the second polo box.

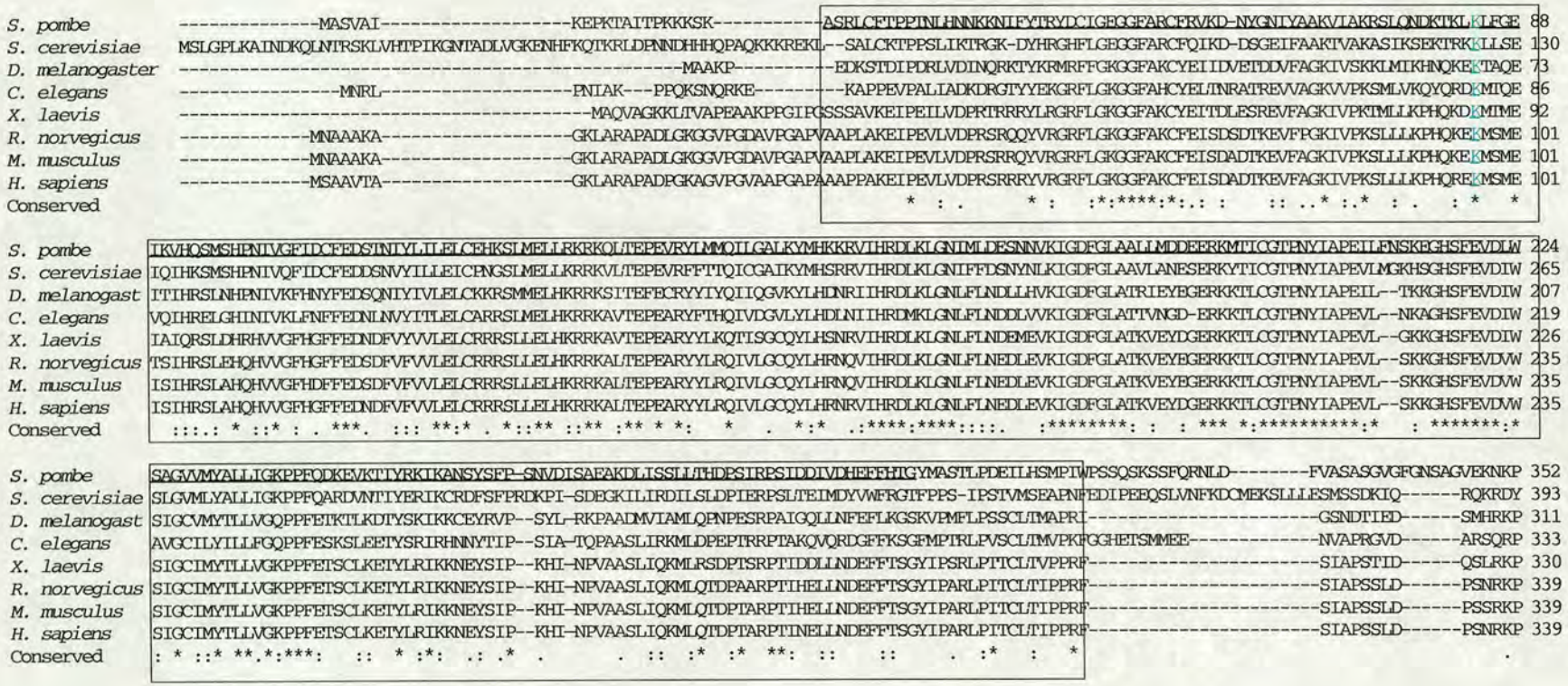

Figure 1.2. Sequence alignment of Plk kinase domains. The above shows an alignment of the kinase domains of Polo-like kinases from various model organisms, Plo1 in *S. pombe* (NP_593647), Cdc5 in *S. cerevisiae* (NP_013714), Polo in *D. melanogaster* (NP_524179), Plk-1 in *C. elegans* (NP_741244), Plx1 in *X. laevis* (AAC60017), Plk in R. *norvegicus* (NP_058796), Plk in *M. musculus* (Q07832) and Plk1 in *H. sapiens* (AAP36458). The kinase domain is boxed.

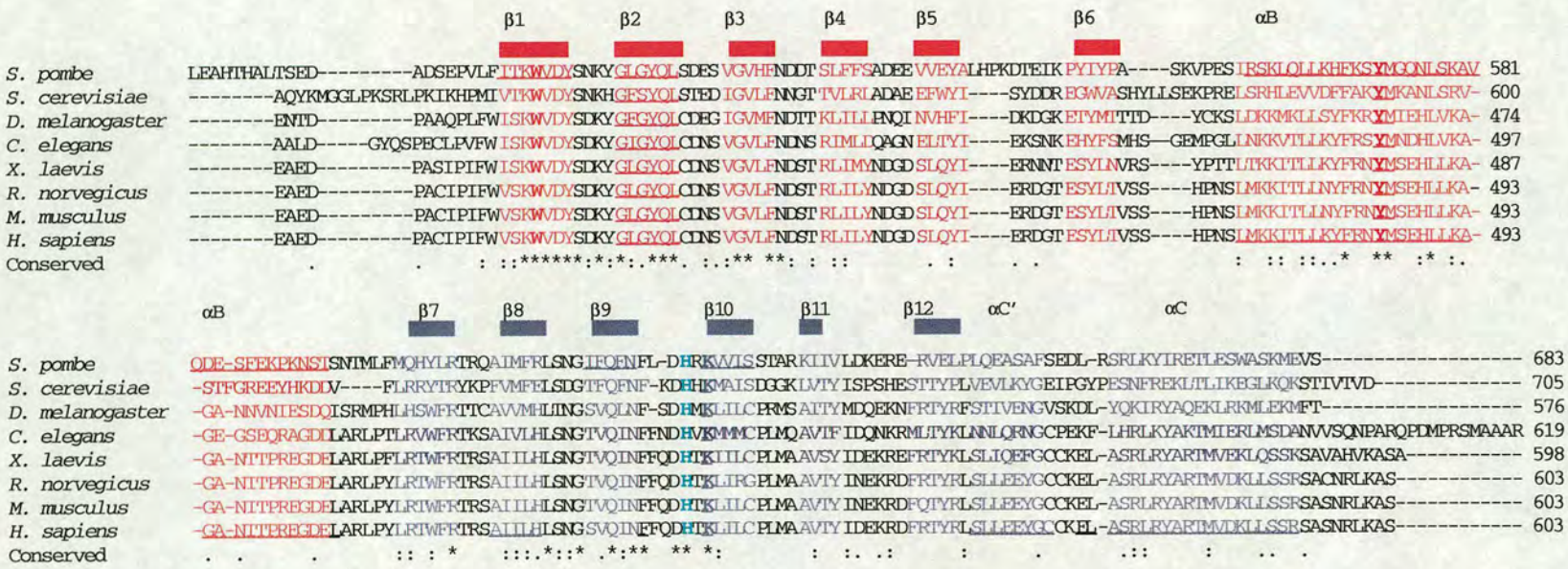

Figure 1.3. Sequence alignment of polo box domains. The above shows an alignment of the polo box domains of Polo-like kinases from various model organisms, Plo1 in *S. pombe* (NP_593647), Cdc5 in *S. cerevisiae* (NP_013714), Polo in *D. melanogaster* (NP_524179), Plk-1 in *C. elegans* (NP_741244), Plx1 in *X. laevis* (AAC60017), Plk in R. *norvegicus* (NP_058796), Plk in *M. musculus* (Q07832) and Plk1 in *H. sapiens* (AAP36458). The red lettering indicates the first polo box and an alpha helix, while the blue lettering represents the second polo box and another alpha helix.

Recently, the non-catalytic domain has been crystallised (Cheng et al., 2003; Elia et al., 2003b). The PBD consists of two polo boxes, which form a structure by intramolecular dimerisation, with six beta sheets in each polo box. The polo cap (part of the linker region between the catalytic and non-catalytic domains may stabilise the structure that facilitates interaction. The structure the polo boxes make allow the PBD to sandwich its interactor (Elia et al., 2003b). Plks bind phosphopeptides (Cheng et al., 2003; Elia et al., 2003a; Elia et al., 2003b). The PBD was crystallised with a phosphopeptide interactor after Plk1 was identified to interact with phosphopeptides in a peptide screen (Elia et al.,2003a). Plk1 interacted with phosphopeptides with a stronger affinity than with corresponding unphosphorylated peptides. The polo-binding motif of the phosphopeptide is similar to the Cdc2 phosphorylation consensus sequence S-pT-P or S-pS-P (Elia et al., 2003a). There is a very strong preference for serine at —1, where pT or

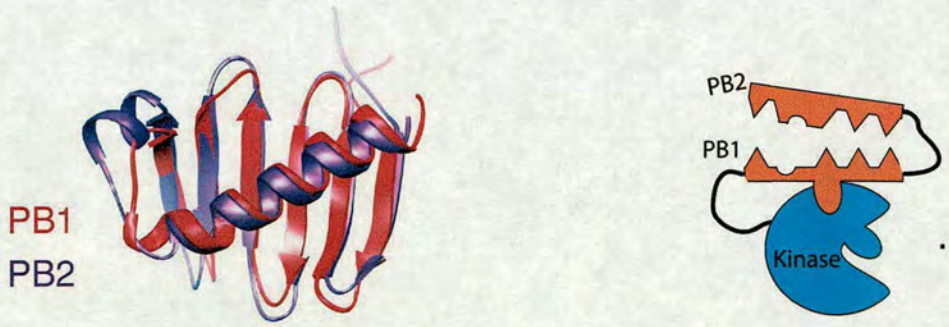

Figure 1.4. Polo box structures. The left shows that two polo boxes, one in red and one in blue, have the same structure. The rights shows that the sandwich structure formed by the polo boxes allow an interacting protein or substrate to be inserted. Adapted from (Elia et al., 2003b).

pS is position 0. Proline at +1 can be substituted by other amino acids, but at this position, hydrophobic amino acids have greater affinity for the polo box domain. *In vivo*, proline is hypothesised to be more prevalent in actual binding motifs of Plk1 interactors, because proline-directed kinases such as Cdk1 and MAPK are most likely to prime a substrate by phosphorylation for interaction with Plk1 (Elia et al., 2003b). Phosphopeptide binding is not restricted to the human species for Plks, but has been shown in *Xenopus* for Plx1 and in budding yeast for Cdc5 (Elia et al., 2003b).

Point mutations in the polo boxes of the budding yeast Cdc5 protein abolish the ability of overexpressed Cdc5 to interact with the spindle poles and to organize cytokinetic structures (Kitada et al., 1993; Song and Lee, 2001), as do point mutations in the PBD of Plo1 in fission yeast (Reynolds and Ohkura, 2003). However, the Plo1 PBD (amino acids 313-684) are sufficient to localise to the spindle pole body (Reynolds and Ohkura, 2003), and the PBD of human Plk1 is sufficient to localise to the centrosome (Elia et al., 2003b). If an optimal phosphopeptide is bound to the PBD, Plk1 cannot localise to the centrosomes (Elia et al., 2003b). The crystal structure has revealed key residues that interact with the phosphopeptide. W414 in the first polo box is positioned across from H538 and K540 in the second polo box to form hydrogen bonds with the phosphate group of a serine or threonine (Elia et al., 2003b). Y485 is important for stabilising the interaction by hydrogen bonding with serine at —1.

The residues in human Plk1 shown in Figure 1.6. are conserved in fission yeast Plo1. Mutations in the polo box domain either alter structure or interfere with phosphopeptide binding (Elia et al., 2003b). Mutations in the corresponding residues reflect the requirement for the polo box domain for Polo-like kinase function. In fission yeast, when W497 is mutated, the residue that corresponds to W414, Plo1 function is abolished (Reynolds and Ohkura, 2003).

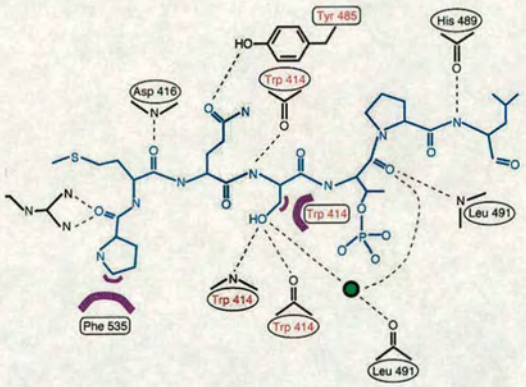

Figure 1.5. Conserved residues interact with the phosphopeptide (Elia et al., 2003b). The phosphopeptde (PMQSpTPL) is shown in blue and the key residues of Plk1 in black. Dotted lines indicate hydrogen bonds, purple crescents represent van der Waals interactions and the green circle is a water molecule. The circled amino acids of Plk1 represent the side chains of residues and the rectangular positions, the main chain.

Similar phenotypes have been obtained for fission yeast Y572 (human Y508), H626 and K628 (human H 538 and K 540) (Reynolds and Ohkura, 2003). Thus, conserved residues preserve the structure of the PBD or maintain phosphopeptide binding.

1.3. Polo-like kinase functions are associated with cell cycle dependent localisation

The structure of the polo box domain is necessary for polo-like kinase function. The PBD is responsible for centrosomal localisation of Plk1 (Elia et al., 2003b) and is thought to localise Plks to mitotic structures (Liu et al., 2004). Mutations in polo boxes of Cdc5 render it incapable of rescuing a budding yeast temperature-sensitive mutant (*cdc5-1*) at the non-permissive temperature (Kitada et al., 1993). Further experiments on human polo-like kinase, Plk1, show that Plk1 can rescue *cdc5-1*, but not if mutated in conserved sequences of the polo boxes (Lee et al., 1998). Localisation at a specific cellular structure as the SPB, or centrosome, probably restricts the pool of interacting proteins for Plks to bind. In turn, the interaction with proteins would mediate Plks functions by initiating certain pathways that activate specific effector proteins.

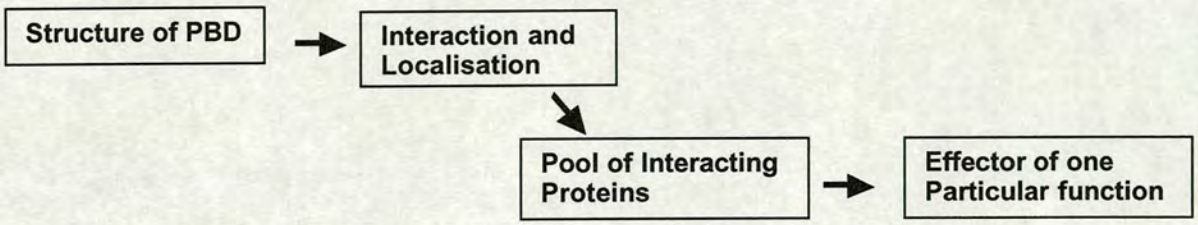

Figure 1.6. The effects of interaction and localisation. The structure of the PBD allows for localisation to centrosomes in human cells and also promotes protein interaction, which activates a pathway to complete a function.

Plks have multiple functions and are key regulators of the cell division cycle. They are involved in each stage of mitosis, playing roles in ensuring the accurate timing of essential processes. As mentioned in section 1.1, the cell cycle has a phase of DNA synthesis, S phase, and another phase of DNA separation through the process of mitosis in M phase, as well as two gap phases (G1 and G2). During mitosis, controls exist to ensure that early processes are completed prior to the initiation of subsequent events (Blagden and Glover, 2003). For example, chromosome alignment precedes chromosome segregation, otherwise aneuploidy results. Prophase is the first stage, in which chromosomes condense, the nuclear envelope breaks down (in higher eukaryotes only) and the spindle, the apparatus which segregates sister chromatids, is assembled (Alberts et al., 2001). Metaphase is the next stage. In metaphase,the spindle fibres are attached to kinetochores of sister chromatids and the chromosomes are aligned at the equator of the cell (Alberts et al., 2001). The spindle assembly checkpoint does not allow cells to progress to anaphase until all the chromatids are attached to spindle fibres (microtubules). Attachment is to the two microtubule organising centres that are opposite one another (Minshull et al., 1994). The spindle assembly checkpoint is composed of various proteins that inhibit activation of another group of proteins, referred to as the anaphase-promoting complex (Fang et al., 1998). Once the inhibition is lifted, cells can progress to anaphase. Anaphase A is the stage where the connection between sister chromatids is severed and in Anaphase B, the spindle elongates to pull the sister chromatids apart. Telophase is defined as the end stage and is characterised by spindle disassembly, chromosome decondensation and

reformation of the nuclear envelope. Cytokinesis is the process where a cell membrane or cell wall is made to form two daughter cells (Alberts et al., 2001).

The localisation of Plks correlates with progression through the cell cycle and is shown in various organisms. (Golsteyn et al., 1995; Golsteyn et al., 1994; Mulvihill et al., 1999). This localisation may control Plk regulation of the cell cycle. At the beginning of mitosis, Plks localise to microtubule organising centres, spindle pole bodies in lower eukaryotes and centrosomes in higher eukaryotes. They can be observed along the fibres (microtubule bundles) of the spindle at times also(B hler et al., 1998; Moutinho-Santos et al., 1999). During early and late anaphase, Plks localise to the spindle midzone after which Plk staining either becomes diffuse in fission yeast, with occasional signal at a ring at the equator of the cell where a new septum will form (B hler et al., 1998; Mulvihill et al., 1999). In human cells, Plk1 can be seen at the midbody at the end of mitosis during cytokinesis (Jang et al., 2002; Lee et al., 1998; Lee et al., 1999; Lee et al., 1995; Logarinho and Sunkel, 1998).

Localisation of Plks at specific mitotic structures allows Plks to interact with a smaller pool of substrates and effectors. Plk localisation is not only associated with phases of the cell cycle, but certain functions require Plk localisation for progression through mitosis. Plks regulate entry into mitosis and their localisation to the spindle pole body or centrosome is necessary to enter mitosis (Grallert and Hagan, 2002; MacIver et al., 2003). Plks are required for anaphase to begin, as they interact with and activate the anaphase-

promoting complex (Nigg, 1998; Shirayama et al., 1998). During early and late anaphase, Plks localise to the spindle midzone and this localisation is required for proper cytokinesis (Golsteyn et al., 1995; Golsteyn et al., 1994; Neef et al., 2003). Thus, localisation of Plks controls access to interactors and subsequent effects.

1.4. The multiple roles of Plks

Plk interactors mediate the multiple roles Plks play. Polo-like kinases promote entry into mitosis by functional interactions between Plks and Cdk pathways (Descombes and Nigg, 1998; Kumagai and Dunphy, 1996; Nigg, 1998; Nigg et al., 1996). Plks also are required during metaphase for the spindle assembly checkpoint (Mulvihill and Hyams, 2002), bipolar spindle formation (Llamazares et al., 1991; Ohkura et al., 1995; Sunkel and Glover, 1988) and they activate the anaphase-promoting complex (Shirayama et al., 1998). In addition to encouraging the commencement of anaphase, Plks are important for mitotic exit (Visintin et al., 2003), the spindle orientation checkpoint (Gachet et al., 2001) and for cytokinesis also (Carmena et al., 1998; Ohkura et al., 1995; Park et al., 2003). Also in meiosis, polo-like kinases are responsible for the monopolar attachment of kinetochores of sister chromatids, so homologous chromosomes can be separated (Lee and Amon, 2001; Lee and Amon, 2003). For one kinase to perform such diverse and multiple roles is a puzzle. The hypothesis presented in this thesis is that Polo-like kinases interact with other proteins for its functions to be mediated.

1.5. Plks promote entry into mitosis

MPF activation triggers entry into mitosis. Plks regulate the phosphatase that activates MPF and cyclin B, one component of MPF (Abrieu et al., 1998; Ellinger-Ziegelbauer et al., 2000; Karaiskou et al., 1999; Kumagai and Dunphy, 1996; Liu et al., 2004; Qian et al., 1998; Qian et al., 1999; Qian et al., 2001; Roshak et al., 2000). Upon entry into mitosis, MPF is rapidly activated by a positive amplification loop, inducing mitotic events such as nuclear envelope breakdown in higher eukaryotes, SPB/centrosome separation and bipolar spindle formation. As mentioned in section 1.1, MPF is maturation promoting factor which is made up of cyclin B and a corresponding cyclin-dependent kinase (Cdk). The components of MPF and its regulators were characterised in fission yeast. Cdc2 is the Cdk of MPF. Cdc25 is a phosphatase that dephosphorylates Cdc2 at T14 and Y15 (Murray and Hunt, 1993). When Cdc2 is dephosphorylated at T14 and Y15, it is activated. Active MPF further enhances Cdc25 activity. Wee1 on the other hand is a kinase that phosphorylates Y15 of Cdc2 (T18 and Y19 in Cdc28), decreasing MPF activity. When MPF is active, it inhibits Wee1 activity (Murray and Hunt, 1993). Myt1 is a kinase that phosphorylates both T14 and Y15 to inactivate MPF (Mueller et al., 1995).

Plks regulate entry into mitosis. Injection of human Plk1 antibodies showed that Plk1 is required for entry into mitosis. Decrease of available Plk1 in nontransformed human cells results in G2 arrest due to defective centrosome maturation (Lane and Nigg, 1996). In addition, Plks act on a regulator of MPF. In *Xenopus*, Plx1 was shown to activate the positive regulator of MPF, Cdc25 (Abrieu et al., 1998; Kumagai and Dunphy, 1996), in turn promoting mitotic commitment. Purified or recombinant Plx1 can phosphorylate Cdc25 *in vitro*, causing Cdc25 to be more immunoreactive to MPM-2 (Kumagai and

Dunphy, 1996). Human Plk1 can phosphorylate recombinant or endogenous Cdc25C, which dephosphorylates Cdc2 resulting in MPF activation (Roshak et al., 2000). On the other hand, Plx1 mRNA with a mutation that abolishes kinase activity is injected into frog eggs, entry into mitosis is delayed by 2.5 hours because phosphorylation of Cdc25C is diminished due to the inactive kinase competing with endogenous Plx1 for binding with Cdc25C (Liu et al., 2004).

Plks promote mitotic commitment by acting on MPF directly, besides affecting Cdc25C, a regulator of MPF. In *Xenopus*, and in humans respective Plks phosphorylate cyclin B (Qian et al., 1999; Yuan et al., 2002). Initially, it was thought that the phosphorylation by Plk1 alters cyclin B1 so that cyclin B1 could be imported into the nucleus (Toyoshima-Morimoto et al., 2001), as mutation of serine to glutamate impedes nuclear translocation (Yang et al., 2001). However, more recent evidence in humans indicates that phosphorylation of cyclin B1 by Plk1 does not cause nuclear import of cyclin B1 (Jackman et al., 2003). However, multiple phosphorylation of serines by the combinatorial action of distinct kinases most likely promotes nuclear localisation of cyclin B1.

Thus, Plks regulate the G2/M transition by promoting centrosome maturation, and phosphorylating Cdc25, as well as by phosphorylating cyclin B. Phosphorylation of Cdc25 results in increased phosphatase activity, which in turn activates MPF. Plk

phosphorylation of cyclin B promotes its nuclear translocation in combination with other kinases, so cyclin B can interact with Cdc2 promoting Cdc2 kinase and MPF activity.

1.6. Plks are required for spindle formation

Plks were originally identified with mutations that resulted in monopolar spindles and abnormal spindles in *Drosophila* (Sunkel and Glover, 1988). Plo1 in fission yeast (Ohkura et al., 1995) and Plx1 in *Xenopus* are are also required for bipolar spindle formation. Both null and conditional mutants of Plo1, the Plk in fission yeast, had phenotypes of monopolar spindles and overcondensed chromosomes (Ohkura et al., 1995; Reynolds and Ohkura, 2003). Antibodies to Plx1 or injection of kinase inactive Plx1 mRNA (Liu et al., 2004) also resulted in the formation of monopolar spindles. Thus, Plks are required for bipolar spindle formation.

The spindle is the cell s machinery to separate duplicated chromosomes and for a bipolar spindle to form, the microtubule organising centres must duplicate, separate and reposition, as well as mature. Microtubules are nucleated with γ-tubulin from the organising centres (Oakley et al., 1990), from where dimers of α- and β-tubulin can polymerise (Heald et al., 1997). Microtubules have directionality with plus and minus-ends. The plus end grows more quickly and the addition of tubulin dimers occurs here more frequently. Microtubules have dynamic instability, growing and shrinking continuously (Alberts et al., 2001). The minus-end is associated with a microtubule

organising centre. Microtubule assembly is facilitated by interaction with microtubule-associated proteins (MAPs) (Ohkura et al., 2001). MAPs can stabilise microtubules. In addition to MAPs, microtubule motors can move along microtubules by using ATP hydrolysis for energy, thus facilitating molecular transport (Vale, 2003). Kinesin is a minus-end motor that moves towards the minus-end of microtubules, or towards the SPB/centrosome. Dynein is a plus-end motor, which moves to the growing end of microtubules. In addition to microtubule organising centres nucleating microtubules forming asters, and MAPs and microtubule motors, there are proteins that allow microtubules to interdigitate such as Cut7 in fission yeast (Hagan and Yanagida, 1990; Hagan and Yanagida, 1992) and TPX2 in frogs (Kufer et al., 2002). TPX2 stabilises overlapping antiparallel microtubule arrays and when TPX2 is depleted from frog egg extracts, microtubules can grow from the centrosome to form asters, but not a spindle (Gruss et al., 2002).

Plks interact with a number of different proteins that are involved in different aspects of spindle formation. Plk interactors include microtubule motors, MAPs and centrosomal proteins, including NudC (Aumais et al., 2003; Zhou et al., 2003), a component of dynein microtubule motor, a microtubule-associated proteins DMAP-85 in *Drosophila* (Cambiazo et al., 2000), Op18 in *Xenopus* (Budde et al., 2001) and TCTP in human cells (Yarm, 2002). Although Plk1 interacts with NudC, it seems not to be necessary for bipolar spindle formation, but microtubule organisation at the midzone during telophase (Aumais et al., 2003; Zhou et al., 2003). Polo is the most likely candidate to

phosphorylate DMAP-85, inhibiting its microtubule-stabilising activity. On the other hand, Plx1 phosphorylates Op18 and reduces Op18 destabilising activity (Budde et al., 2001). Plk1 phosphorylates TCTP, but if not phosphorylated the centrosomes are more diffuse (Yarm, 2002). Thus, it appears that phosphorylation by Plks alters microtubule dynamics. This may influence spindle elongation and the general dynamics of microtubule growth and shrinkage. In terms of Plk interactions, the effector functions known are for the centrosome-associated proteins. Polo phosphorylates Asp, a protein that sits on the inner face of the centrosome (the nuclear side) (do Carmo Avides et al., 2001). When Asp is phosphorylated, centrosome maturation, aster formation and focussing of spindle poles occur (do Carmo Avides et al., 2001). In fission yeast, Plo1 must interact with Cut12 and Fin1 kinase to form a spindle (Grallert and Hagan, 2002; MacIver et al., 2003). Plk1 interacts with Nlp1 in human cells, a centrosome-associated protein (Casenghi et al., 2003). Nlp1 associates with the γ-tubulin of the centrosome, but when Nlp1 is phosphorylated by Plk1, Nlp1 no longer associates with γ-tubulin at the centrosomes (Casenghi et al., 2003). Then, a bipolar spindle forms. However, if Nlp1 is mutated so that it cannot be phosphorylated, a bipolar spindle cannot be formed (Casenghi et al., 2003).

1.7. Plks regulate exit from mitosis

After the spindle is assembled, the sister chromatids are separated in anaphase. The spindle assembly checkpoint prevents separation of DNA until all chromosomes are aligned and

attached to opposite poles by microtubules (Fang et al., 1998). Then the inhibition of the anaphase promoting complex is lifted (Fang et al., 1998; Yanagida et al., 1999). Plo1 is required for an active spindle assembly checkpoint in fission yeast (Mulvihill and Hyams, 2002). Plks are also thought to activate the anaphase promoting complex. The APC is a ubiquitin ligase of which there are two forms, to target substrates for degradation by the 26S proteasome (Shirayama et al., 1998). The first form of the APC contains Cdc20, which has specificity for securin, an inhibitor of separase (Shirayama et al., 1998). Separase is an enzyme that cleaves cohesin (the complex of proteins that join the sister chromatids together). In budding yeast, Cdc5 phosphorylates a cohesin component so that it is more efficiently cleaved (Alexandru et al., 2001). Thus, anaphase A characterised by severing of the cohesion between sister chromatids. The second form of the APC has a different specificity factor, Cdh1, that leads to the destruction of Cdc5 and Clb2 (budding yeast cyclin B) (Shirayama et al., 1998). The degradation of cyclin B leads to the inactivation of maturation promoting factor (MPF). *cdc5* mutants cannot exit mitosis, due to persistent levels of cyclins, Clb2 (Charles et al., 1998). Hence, anaphase B is characterised at a cellular level by spindle elongation and biochemically, by cyclin degradation.

The phosphorylation and dephosphorylation of specific components of the APC allow it to have activity, regulating precise events with accurate timing (Kotani et al., 1998). Plks play an important role in activating the APC and exit from mitosis. In *Xenopus*, a metaphase arrest was also observed when Plx1 was immunodepleted, but exit from mitosis could be restored upon addition of recombinant Plx1 (Descombes and Nigg, 1998). Overexpression of *plx1* mRNA encoding inactive kinase blocks activation of APC/cyclosome (Liu et al., 2004). Hence, the polo box domain is required for the metaphase/anaphase transition. It may be that Plx1 inactive kinase competes with

endogenous Plx1 for Cdk1-phosphorylated sites and without further kinase activity from a Plk, the APC cannot be fully activated. Thus, Plks are required for activation of the anaphase-promoting complex.

In addition to the APC, two networks of proteins act later in the cell cycle to ensure progression through anaphase. The two networks are referred to as FEAR and MEN. Both FEAR and MEN promote the release of a phosphatase, Cdc14, that reverses phosphorylation events put in place by MPF (Bardin and Amon, 2001). The dephosphorylation of substrates allows for mitotic exit.

FEAR initiates release of Cdc14 and MEN maintains Cdc14 out of the nucleolus. FEAR, standing for Cdc14 (fourteen) early anaphase release, and consisting of Cdc5, separase (Esp1), Slk19 a kinetochore-associated protein and Spo12 initiates the release of Cdc14, a nucleolar protein (Cohen-Fix, 2003; Stegmeier et al., 2002). Cdc5 is required for anaphase progression, not only for its action as a component of FEAR, but also because Cdc5 phosphorylates Net1, a nucleolar protein that maintains Cdc14 in the nucleolus (Visintin et al., 2003). The action of Cdc5 on Net1 encourages Cdc14 nucleolar release.

The second network of genes, the mitotic exit network (MEN) maintains the released state of Cdc14 (Figure 1.8). Cdc5 interacts with a component of MEN (Stegmeier et al., 2002). MEN is also a spindle orientation checkpoint in budding yeast, as it is not activated until the mother SPB enters the daughter cell or bud, ensuring the bipolar spindle is oriented correctly to separate chromosomes into two separate daughter cells (Bardin and Amon, 2001; Geymonat et al., 2002; McCollum and Gould, 2001). Nud1 anchors the components of the MEN to the SPB (Simanis, 2003a; Simanis, 2003b).

Figure 1.7

Tem1 is part of the MEN, which is a GTPase, which acts as a molecular switch. Lte1 is the exchange factor to replace GTP when it has been hydrolysed to GDP (Shirayama et al., 1994). Bub2 and Bfa1 form a complex that localises asymmetrically to one SPB moreso than the other and act as a GTPase activating protein (Figure 1.8). This means that at the SPB where Bub2 and Bfa1 are more concentrated, Tem1 is in its GDP-bound form and MEN is not active at this SPB (Simanis, 2003a; Simanis, 2003b). Cdc5 interacts with Bfa1 and phosphorylates it, inhibiting the complex's GTPase activity and increasing MEN activity (Geymonat et al., 2003). The SPB that enters the bud has higher levels of Tem1 in its GTP-bound form (Figure 1.8). Tem1-GTP activates Cdc15, which in turn signals to Dbf2/Mob1 (Lu et al., 2002; Pereira et al., 2002). The end effect is for Cdc14 to be maintained out of the nucleolus MEN (Stegmeier et al., 2002).

Budding yeast MEN shares a number of similarities with fission yeast SIN (Septation initiation network), which will be discussed in next section 1.8. on cytokinesis. The SIN regulates septum formation and transmits signals of the GTPase Spg1 to the equatorial ring (Schmidt et al., 1997). They both use a GTPase to act as a molecular switch to delay exit from mitosis in the case of MEN, or to delay septation in the case of SIN (Bardin and Amon, 2001, Figure 1.8). For both systems, the delay is to ensure that sister chromatid segregation has taken place, prior to cell division by cytokinesis. The Plks, Cdc5 and Plo1, function at the top of the cascades MEN and SIN, respectively (Simanis, 2003b; Tanaka et al., 2001).

1.8. Cytokinesis

Plks are required for cytokinesis in humans (Aumais et al., 2003; Neef et al., 2003), *Drosophila* (Carmena et al., 1998), budding (Song et al., 2000; Song and Lee, 2001) and fission yeast (Ohkura et al., 1995; Reynolds and Ohkura, 2003). In *Drosophila*, Polo associates with a kinesin-like protein called Pavarotti (Adams et al., 1998). Mutants of *pav-KLP* have enlarged, multinucleate cells result from the failure to form a cleavage furrow (Adams et al., 1998). In *polo* mutants, Pavarotti and Peanut (a septin) are not correctly localised and actin is not incorporated into the contractile ring (Carmena et al., 1998). Thus, cytokinetic proteins require Polo for appropriate localisation.

When NudC, a dynein heavy chain, is depleted or overproduced in human cells, Plk1 is mislocalised and elongated, multinucleate cells form (Aumais et al., 2003). The microtubules found in the midzone are disorganised. Plk1 interacts with and phosphorylates NudC (Zhou et al., 2003). Ectopic expression of wild-type NudC can rescue NudC depleted cells, but NudC lacking Plk1 phosphorylation sites cannot (Zhou et al., 2003). Hence, microtubule organisation not only facilitates Plk1 localisation, but Plk1 acts on substrates to alter microtubule dynamics and subsequently cytokinesis.

Plk1 phosphorylation is required for cytokinesis in human cells (Neef et al., 2003). In *Drosophila*, Pav-KLP is important to mediate Polo cytokinetic functions. Pav-KLP is also known as Mklp1 in mice (Lee et al., 1995). In human cells, a protein related to Mklp1, Mklp2, is a target for Plk1. Once phosphorylated, Mklp2 binds the polo box domain with more affinity (Neef et al., 2003). The tighter binding directs Plk1 to the central spindle. Antibodies to Mklp2 that prevent phosphorylation by Plk1 have a cytokinetic defect (Neef et al., 2003). There is an interdependent relationship between Plk1 localisation, phosphorylated substrate binding and microtubule organisation.

1.9. Meiosis

Plks are required for severing the connection between homologous chromosomes at anaphase I. In budding yeast, Cdc5 ensures that the kinetochores of chromosomes become attached to the same spindle pole body in meiosis, rather than to the two opposite SPBs as in mitosis (Watanabe, 2003). This is referred to as monopolar attachment, where kinetochores are oriented towards one pole so that sister chromatids migrate to that pole and homologous chromosomes are separated. The homologous chromosomes are held together by a meiotic-specific cohesin, called Rec8, Scc1, Smc1 and Smc3 (Klein et al., 1999). Cdc5 is critical for cleaving Rec8 and removal of distal cohesin to resolve recombination events (Clyne et al., 2003). Then, calls can proceed through anaphase I (Lee and Amon, 2001; Lee and Amon, 2003) for separation of homologous chromosomes.

Cdc5 is a component of the FEAR network, which regulates anaphase progression in conjunction with other signals (Visintin et al., 2003) (see section 1.7. on exit from mitosis). FEAR has a plays an important role in meiosis (Stegmeier et al., 2002), because cyclin B is not expressed at high levels in budding yeast meiosis (Lee and Amon, 2003). Cells lacking a component of FEAR/Cdc14 can only undergo meiosis I (Lee and Amon, 2003). Moreover, some cells go through reductional division rather than equational division (where sister chromatids are segregated, rather than homologous chromosomes being separated). FEAR seems to be required for spindle disassembly during meiosis I also (Lee and Amon, 2003). Thus, the budding yeast Plk Cdc5 is important for kinetochore orientation and progression through meiosis I so two consecutive divisions can take place.

1.10. Rationale, Hypothesis and Aims: As one can observe, Plks drive multiple diverse functions in the cell cycle. The interactions Plks form permit them to initiate distinct pathways that lead to different cellular events (entry into mitosis, spindle formation, sister chromatid segregation, exit from mitosis, cytokinesis and meiosis). Considering the number of Plk interactors that are associated with cellular functions, the hypothesis of this thesis is that Polo-like kinases interact with other proteins for their functions to be mediated. Searching for interactors that mediate Plk function is the first aim of this thesis.

Table 1.2. The interactors of polo-like kinases and associated cell cycle functions

Associated Function	Species	Interaction
Entry into mitosis	Fission yeast	Plo1-Cut12
	Budding yeast	Cdc5-Swe1 (Wee1)
	Frog	Plx1-Cdc25*
	Human	Plk1-Cdc25C*
	Human	Plk1-Myt1*
Position of medial ring	Fission yeast	Plo1-Dmf1
Bipolar spindle formation		
Microtubule stability	Frog	Plx1-Op18
	Fruitfly	Polo-DMAP-85
	Human	Plk1-TCTP*
	Human	Plk1-Nlp1
Centrosome/Pole focus	Fruitfly	Polo-Asp
Exit from mitosis	Budding yeast	Cdc5-Bfa1*
	Budding yeast	Cdc5-Net1*
Cytokinesis	Fruitfly	Polo-PavKLP
	Mouse	Plk-Mklp1
	Human	Plk1-Mklp2*
	Human	Plk1-NudC*

*-Plk-interacting proteins that are known substrates or strong evidence exists that they are Plk substrates. Xenopus Cdc25 (Kumagai and Dunphy, 1996; Qian et al., 2001), human Cdc25C (Roshak et al., 2000), Myt1 (Nakajima et al., 2003), TCTP (Yarm, 2002), Bfa1 (Geymonat et al., 2003; Hu et al., 2001; Park et al., 2003; Pereira et al., 2002), Net1 (Yoshida and Toh-e, 2002), Mklp2 (Neef et al., 2003), (Zhou et al., 2003).

It is important to study an organism in which there is evidence for the multiple roles to understand if it is different interacting proteins that mediate the distinct functions of Plks. In budding yeast does spindle formation occurs in S phase and Cdc5 depletion does not affect the spindle. Cdc5 depletion does affect cytokinesis and meiosis, however, these events occur in different cell cycles. How one protein can regulate many events within one cell cycle is the question. Entry into mitosis, spindle formation, the metaphase-anaphase transition and septation or cytokinesis were the roles that are affected simultaneously by Plks. Meiosis occurs at a different time. In human cells, bipolar spindle formation is affected when Plk1 is depleted due to defects in centrosome amplification and maturation (Lane and Nigg, 1996), while in *Xenopus* eggs, entry into mitosis and exit from mitosis are affected. Although *Xenopus* is a powerful model for biochemical studies of interaction, a kinase interacts so transiently that it is difficult to use biochemical techniques to assay interaction. In *Drosophila* and fission yeast, spindle formation, the metaphase-anaphase transition and cytokinesis fail to occur properly when Polo or Plo1 are mutated (Glover et al., 1996; Ohkura et al., 1995; Reynolds and Ohkura, 2003).

The second stipulation would be to study an organism that has only one polo-like kinase to be able to find whether it is different interacting proteins that mediate separate functions. Budding yeast, fission yeast and *Drosophila* are the model organisms that have only one Plk. Although *Drosophila* has spindle formation and cytokinetic defects and is a good organism to study genetics, it is difficult to identify mutants, genetic interactions and new proteins quickly, as one cannot identify mutations as readily as in yeast. Fission yeast is a genetically tractable organism that has been sequenced almost fully. Cells with mutations in *plo1*, the fission yeast polo-like kinase gene, exhibit a pleiotropic phenotype. The cells have monopolar spindles, metaphase arrest and

septation defects (Ohkura et al., 1995). Hence, the diverse functions of Polo-like kinase, such as bipolar spindle formation, metaphase-anaphase transition and cytokinesis are observed within one organism.

1.11. General information about fission yeast

Fission yeast has been used extensively in cell cycle studies. Since fission yeast is rod-shaped, grows lengthwise and the divides in the middle, its length is a good indicator of progression through the cell cycle. Fission yeast is a simple single-celled eukaryote, non-filamentous *Ascomycete* fungus with many of the features found in cells of more complicated eukaryotes. For example, during cell division an equatorial contractile ring of actin and myosin forms (Gould and Simanis, 1997). This is referred to as the cytokinetic actomyosin ring (CAR) and is assembled at the beginning of mitosis (Mulvihill and Hyams, 2002). During cytokinesis, CAR constriction takes place, but unlike higher eukaryotes, a new septum is formed to separate daughter cells. Fission yeast diverged from budding yeast ~330-420 million years ago and from metazoa and plants 1.1-1.2 billion years ago (www.sanger.ac.uk/ Project/S_pombe/orginfor.shtml). *Schizosaccharomyces pombe* is a well-characterised organism with 1,200 genes having been functionally characterised and 4,824 genes being known (Wood et al., 2002) (www.sanger.ac.uk/Project/ S_pombe/orginfor.shtml). The genome of fission yeast consists of 13.8 Mb of DNA carried on three chromosomes, 99.9%, of which is sequenced (Wood et al., 2002).

1.12. Role of Plks in fission yeast

In fission yeast, Plo1 has cell cycle dependent localisation patterns (B hler et al., 1998; Mulvihill et al., 1999), which may control Plo1 and its consequent multiple functions (Glover et al., 1996). The following sections describe localisation and interactors that mediate these diverse functions of Plo1.

1.12.a. Plo1 localisation in the fission yeast cell cycle

Plo1 localisation has been studied for its effects on the cell cycle and for what regulates Plo1 localisation. The localisation of Plo1 has been shown by staining with antibodies and with live imaging (B hler et al., 1998; Mulvihill et al., 1999), showing that during interphase the signal is dispersed. Upon entry into mitosis, Plo1 localises to the SPB and stays on the SPBs as they separate to form the bipolar spindle. Plo1 is detected on the spindle faintly (B hler et al., 1998; Mulvihill et al., 1999). Plo1 is seen on the SPBs as the spindle elongates, until anaphase B, when the signal dissipates (B hler et al., 1998; Mulvihill et al., 1999). Occasionally, a faint cortical ring is observed (B hler et al., 1998). Plo1 protein levels are constant throughout the cell cycle (Mulvihill et al., 1999), so the accumulation of Plo1 at the SPB is not due to changes in amounts of Plo1 protein expressed; nor is the disappearance of Plo1 from the SPB.

Whether the SPB localisation of Plo1 is dependent on microtubules has not been completely investigated. Incubation at 4°C does not disrupt SPB localisation of Plo1 (Mulvihill et al., 1999), but thiabendazole (a microtubule-destabilising agent) treatment is reduced at the SPB (Mulvihill and Hyams, 2002). Plo1 localisation to the SPB does rely on the activity of maturation promoting factor (MPF). MPF is the activator of mitosis and is comprised of a cyclin-dependent kinase, Cdc2 (Beach et al., 1982), and a regulatory protein called cyclin B (Beach et al., 1982; Lohka et al., 1988). The phosphatase Cdc25 dephosphorylates Cdc2, activating the kinase (Forsburg and Nurse, 1991; Moreno et al., 1989). Temperature-sensitive mutants of the MPF component Cdc2 and the MPF activator Cdc25 have been used to assess the requirement of MPF activity for SPB localisation of Plo1 (Mulvihill et al., 1999). Plo1 does not localise to the SPB in *cdc25-22* and *cdc2-33* at the restrictive temperature (Mulvihill et al., 1999), indicating the Plo1 localisation to the SPB is dependent on the presence of active MPF.

Plo1 localises to the SPB and interacts with Cut12, a protein that associates with the SPB and promotes entry into mitosis (MacIver et al., 2003). The *cut12-s11* gain-of-function mutant (originally called *stf1-1*, suppressor of twenty-five) suppresses the lethality of *cdc25-22*, by promoting entry into mitosis while bypassing the requirement for active Cdc25 (Bridge et al., 1998). When *cut12-s11* mutant cells enter mitosis and Plo1 is localised to the SPBs at the beginning of mitosis (Mulvihill et al., 1999). Plo1 may need to be maintained at the SPB for its further functions. Overexpression of a checkpoint protein, Dma1, prevents Plo1 from localising to the SPB after mitosis has begun (Guertin

et al., 2002). Thus, proteins that regulate progression through the cell cycle, such as MPF-associated proteins Cdc2 and Cdc25, and spindle assembly checkpoint proteins (Dma1) affect Plo1 localisation.

The polo boxes are essential for SPB localisation of polo-like kinases as noted in section 1.3. SPB localisation of Plo1 is essential for cell survival. Extensive study in fission yeast has shown that the polo boxes when mutated or deleted cannot localise Plo1 to the SPB and furthermore, cannot rescue a *plo1* deletion strain (Reynolds and Ohkura, 2003). On the other hand, the C-terminal non-catalytic domain containing the polo boxes along with the linker between the catalytic and non-catalytic domains is sufficient for cell cycle dependent localisation of Plo1 (Reynolds and Ohkura, 2003).

1.12.b. Plo1 interacts with Cut12 to promote entry into mitosis

The interaction of Plo1 with Cut12 promotes entry into mitosis. A mutation in Cut12, *stf1-1/cut12-s11* , suppresses the conditional lethality of the temperature-sensitive yeast mutant *cdc25-22* (Bridge et al., 1998). *cdc25-22* cells are arrested in G2 at higher temperatures because this form of Cdc25 cannot activate MPF and mutant cells eventually die. The double mutant of *cdc25-22* and *cut12-s11* can survive. However, Cut12 cannot rescue the mutation in Cdc25 without Plo1 protein. A truncated protein that lacks the polo boxes was used to make a triple mutant, *cut12-s11 cdc25-22 plo1-ts19*. The triple mutant could not survive at the restrictive temperature, whereas the double mutant of *cut12-s11 cdc25-22*

could grow (MacIver et al., 2003). Hence, Plo1 mediates the rescue of *cdc25-22* by *cut12-s11*.

In the *cut12-s11* fission yeast mutant, Plo1 associates with the SPB throughout the cell cycle (Mulvihill et al., 1999). Wild-type fission yeast exhibit SPB-associated Plo1 during mitosis, but not during interphase (Mulvihill et al., 1999). Thus, the *cut12-s11* most likely rescues *cdc25-22* by having Plo1 persistently activating the SPB, promoting entry into mitosis. Further evidence is that *cut12-s11 cdc25-22* cells with a non-functional NimA (never-in-mitosis) related kinase, Fin1, no longer had Plo1 at the SPB and could not suppress *cdc25-22* mutation (Grallert and Hagan, 2002). Elevated levels of Fin1 cause Plo1 to be recruited to the SPB during interphase (Grallert and Hagan, 2002).

There is an interplay and interdependence of the cyclin-dependent kinases and the polo-like kinases. The interaction between Plo1 and Cut12 that allows Cdc25 to mediate entry into mitosis. Evidence for the interaction between Plo1 and Cut12 comes from yeast two hybrid and immunoprecipitation experiments (MacIver et al., 2003), where amino acids 122-325 of Cut12 are necessary and sufficient for interaction with Plo1 (MacIver et al., 2003). This region of Cut12 contains a coiled-coil domain.

Constitutively active Plo1 kinase (S124D/T197D) suppresses the conditional lethality of *cdc25-22* at the non-permissive temperature (MacIver et al., 2003). The gain-of-function mutation in Cut12, *cut12-s11*, enhanced Plo1 kinase activity and mitotic activation of the

SPB, whereas a loss-of-function mutation, *cut12-1*, diminished Plo1 kinase activity. The SPB is more immunoreactive to an epitope, MPM-2, during mitosis (MacIver et al., 2003). A decrease in MPM-2 staining of the SPB, indicating a less mitotically active SPB, was observed in *cut12-1* cells (MacIver et al., 2003). Thus, association of Plo1 with the SPB component, Cut12, enhances Plo1 kinase activity, in turn activating the SPB.

1.12.c. SPB localisation of Plo1 and interaction of Plo1 with Cut23 are required for progression through anaphase

The metaphase-anaphase transition is delayed by the spindle assembly checkpoint. SPB localisation of Plo1 is dependent on Mad2, a component of the spindle assembly checkpoint (Mulvihill and Hyams, 2002). In *plo1*Δ cells, a metaphase arrest is observed in a portion of the cells (Ohkura et al., 1995). Once the spindle assembly checkpoint has been passed, anaphase can progress as the inhibition on the anaphase-promoting complex is lifted.

To find genetic interactions of *plo1*, a chemical mutagenesis screen was carried out to find mutants that depended on high levels of Plo1 (Cullen et al., 2000). In fission yeast, a high *plo1*-dependent mutant, *pld9*, was found to arrest in metaphase with overcondensed, unseparated chromosomes with a short spindle (Cullen et al., 2000; May et al., 2002). *pld9-PD26* mutant could only survive when high levels of Plo1 were expressed. *pld9* is allelic with *cut23*, a gene encoding a component of the anaphase-promoting complex

(APC) (May et al., 2002). The metaphase arrest was due to the failure of cyclin B (Cdc13) and securin (Cut2) to be degraded in *cut23-PD26* cells (May et al., 2002). The phenotypes could be overridden by expression of Plo1 at high levels. Overexpression of Plo1 did not affect APC complex, and the APC appears as if it is intact biochemically, according to sucrose gradients, in the *cut23-PD26* mutant (May et al., 2002).

Cut23 interacts physically with Plo1 by immunoprecipitation assay. The interaction is direct using a GST-Cut23 pulldown of *in vitro* translated Plo1 (May et al., 2002). Tetratricopeptide repeat (TPR) motifs are present in a number of proteins and mediate protein-protein interactions, particularly of multiprotein complexes (www.sanger.ac.uk/Pfam). TPR motifs 2-9 of Cut23 produce stable protein that can interact with Plo1 by immunoprecipitation. TPR motifs 1-8 were required for interaction with Plo1 in yeast two-hybrid assay, and the first 50 amino acids of Cut23 were required for interaction with Plo1 in yeast two-hybrid assays. TPR motifs have a helix-turn-helix arrangement with adjacent TPR motifs packing in a parallel fashion to produce a spiral of repeating anti-parallel α-helices (www.sanger.ac.uk/Pfam). The point mutation in the 5th TPR motif of Cut23 changing serine to asparagine at amino acid 349 causes a 60% reduction for Plo1 interaction as measured by β-galactosidase activity and is responsible for the *pld9* mutant (May et al., 2002). Thus, Plo1 interacts with Cut23, an APC component, genetically and physically, activating it to allow progression through anaphase.

1.12.d. Plo1 drives septation in fission yeast

Plo1 null has septation defects, both in that there is no septum formed and also that no cytokinetic actomyosin ring (CAR) is formed (Ohkura et al., 1995). Thus, Plo1 is required for both CAR formation and septation initiation in fission yeast. In prophase, actin and myosins, and tropomyosin (Cdc8) as well as formins and profilin are assembled into the CAR. Formins, like Cdc12, bind the barbed end of actin and help nucleation, whereas profilin binds formin homology domains to inhibit nucleation (Kovar et al., 2003; Pelham and Chang, 2002).

The CAR is placed by microtubules and a Plo1-interacting protein Mid1/Dmf1 in prophase (B hler et al., 1998; Pardo and Nurse, 2003). After anaphase, the CAR constricts and two septa are formed on either side of the CAR to separate two new daughter cells. Plo1 overexpression drives septation, because Plo1 can initiate CAR formation and septum formation so that septation occurs even during interphase (Ohkura et al., 1995).

Septation initiation takes place after CAR has been formed and placed. The septation initiation network is a group of genes that control septation initiation and coordinate it with chromosome segregation and anaphase progression. The SIN genes are orthologues of the MEN (Bardin and Amon, 2001; Simanis, 2003a; Simanis, 2003b). Both use GTPases to signal switches in a phosphorylation cascade for an end result (Simanis,

2003a; Simanis, 2003b). In the MEN, the end result is nucleolar release of Cdc14, the phosphatase that reverses MPF phosphorylations (Bardin and Amon, 2001). Although the end point of SIN activation is not the same, the Cdc14 homologue Clp1 is released from the nucleolus. It is the translocation of one SIN kinase from the SPB to the CAR or equatorial region, which allows for septation initiation (Balasubramanian et al., 1998; Chang and Gould, 2000; Guertin et al., 2002; Krapp et al., 2003; Krapp et al., 2001; Tomlin et al., 2002). The SIN is comprised of Sid4 and Cdc11, which are SPB proteins that act as anchors for all of the other SIN components (Morrell et al., 2004), Spg1 GTPase and two proteins, Cdc16 and Byr4 complex together to make a GTPase activating protein (Li et al., 2000). No guanine nucleotide exchange factor is known at this time (Simanis, 2003a). Once Spg1 is active, Cdc7 kinase localises at the SPB (Balasubramanian et al., 1998; Schmidt et al., 1997; Sohrmann et al., 1998). Plo1 activation and localisation to the SPB is associated with Cdc7 recruitment to the SPB (Mulvihill et al., 1999). Cdc7 in turn recruits Cdc14 (not the same as Cdc14 phosphatase) in budding yeast and Sid1 kinase (Guertin et al., 2000), which in turn recruits Sid2 kinase and Mob1 (Sparks et al., 1999). When Sid2 is activated, it moves from the SPB to the CAR, which initiates septation (Simanis, 2003a; Simanis, 2003b; Sparks et al., 1999).

Plo1 localisation to the SPB is correlated with sepation, as septation occurs after Plo1 localises to the SPB. Yet septation and SPB localisation of Plo1 are not dependent on one another. There is a strong correlation between SPB localisation of Plo1 and septation.

Table 1.3. The components of the mitotic exit network and septation initiation network

MEN (*Saccharomyces cerevisiae*)	SIN (*Schizosaccharomyces pombe*)
	Sid4
Nud1	Cdc11
Tem1 GTPase	Spg1 GTPase
Bub2/Bfa1 GTPase activating protein	Cdc16/Byr4 GTPase activating protein
Lte1 guanine nucleotide exchange factor	
Cdc15	Cdc7
	Sid1/Cdc14
Mob1/Dbf2	Sid2/Mob1

Septation occurs after Plo1 localises to the SPB in synchronised fission yeast (Mulvihill et al., 1999), where Plo1 functions at the top of the cascade of events that initiate septation (Tanaka et al., 2001). However, SPB localisation of Plo1 is not required for septation to occur, if septation is driven downstream. In *cdc16-116* cells, Cdc16 an inhibitor of septation initiation is mutated (Minet et al., 1979) so septation initiation is uninhibited. At the restrictive temperature in these cells, Plo1 is at the SPB for one round of septation and then, it leaves the SPB (Mulvihill et al., 1999). The cells can still septate without Plo1 at the SPB. Hence, SPB localisation of Plo1 is not required for septation to take place.

On the other hand, if septation is blocked by mutating activators of septation, Plo1 still associates with the SPB. Experiments were performed at the non-permissive temperature in temperature-sensitive strains of septation mutants: *cdc7-A20, cdc11-IH1, cdc14-118, cdc15-140* (Mulvihill et al., 1999), indicating that the dissociation of Plo1 from the SPB is not required for septation. Fission yeast strains can still undergo septation even if the cells have not gone through anaphase. Some *cut* (cell untimely torn) mutants undergo septation in the absence of nuclear division (Samejima et al., 1993). In the *cut* mutants, Plo1 localises to the SPB and does not dissociate from the SPB (Mulvihill et al., 1999). This indicates that it is not necessary for Plo1 to leave the SPB for septation to occur (Mulvihill et al., 1999).

From the screen for high Plo1-dependent mutants, not only were genes involved in metaphase arrest such as *cut23* found, but also septation initiation genes were found. *pld* mutants *cdc11*, *cdc7*, *cdc15*, *spg1* and *sid2* were found to be dependent on high levels of Plo1. Thus, some of the high *plo1*$^{+}$-dependent mutants identified are components of the septation initiation network. These mutants depended on high levels of Plo1 for cell survival (Cullen et al., 2000). Therefore, Plo1 acts in conjunction with the SIN pathway.

Peak kinase activity of Plo1 occurs just prior to septation. Plo1 itself is phosphorylated, but probably not by MPF, because T14A, within a Cdc2 consensus site, can still rescue *plo1*Δ (Tanaka et al., 2001). Plo1 levels are constant; the protein is not degraded unlike

Plks of other species. Thus, the kinase activity is regulated, not the level of protein. Two peaks of activity (Plo1 kinase) and the second peak coincides with the beginning of Cdc13 degradation (Tanaka et al., 2001). When Cdc13 degradation stops, kinase activity returns to basal levels, but at basal levels, Plo1 no longer has the affinity for the SPB (Mulvihill and Hyams, 2002). The inactive kinase Plo1-K69R can localise to the SPB (Reynolds and Ohkura, 2003). When Plo1 interacts, the polo box domain is engaged in the interaction and the conformation changes so that the kinase is free and its activity is higher (Elia et al., 2003b).

Sid4 functions at the top of the SIN by anchoring all components of the SIN, not through directly interacting with those components, but by interaction with the docking protein Cdc11 (Chang and Gould, 2000; Krapp et al., 2001; Morrell et al., 2004; Tomlin et al., 2002). Plo1 kinase activity seems to activate the SIN and septation by the synchronised recruitment of Cdc7 to the SPB after peak Plo1 kinase activity (Mulvihill et al., 1999). In a double mutant of *spg1.B8*, *cdc7.A20* only a slight reduction in Plo1 kinase activity is observed (Tanaka et al., 2001). Thus, Plo1 kinase activity is not affected by defects in the SIN. In this double mutant, Plo1 overproduction could not induce septation. This brings about the question of Plo1 requirement for septation. A strain carrying mutations in *plo1* and in *cdc16* to drive septation, *plo1-ts4 cdc16-116*, was able to septate (Tanaka et al., 2001). This indicates that Plo1 is not required for septation if driven by SIN components and it also demonstrates that Plo1 acts upstream of Cdc16. A triple mutant carrying mutations in *plo1, cdc16* and *sid2*, could not septate, indicating that a functional

SIN is required for Plo1 to drive septation (Tanaka et al., 2001). Thus, Plo1 acts at the top of SIN (Tanaka et al., 2001).

1.12.e. Fission yeast meiosis

Plks regulate meiosis as well and to date studies have shown their importance in getting homologous chromosomes to be separated appropriately. Whether Plks are important for entry into meiosis and spindle formation has not investigated. Little is known about Plo1 during meiosis. *plo1* is one of the genes expressed in the middle of meiosis. The *cdc20* (APC regulator) is an early gene expressed during meiotic S phase at 2 hours after induction of meiosis. *spo12* and *spo13* involved in sporulation at 10 hours after nitrogen starvation are late genes. *plo1* is expressed at higher levels at time point 5-6 hours after induction during the meiotic divisions (Mata and Bahler, 2003). In this laboratory, a meiosis-specific protein, Spo13, was found to interact with budding yeast Plk, Cdc5, which is necessary for separation of homologous chromosomes during meiosis (Lee et al., 2002; Reynolds, 2001; Shonn et al., 2002). The second aim of this thesis was to find meiosis-specific interactors of Plo1.

Chapter 2: Identification of putative Plo1 interacting proteins by a yeast two-hybrid screen

2.1. Search for Plo1 interacting proteins

Since polo-like kinases have diverse roles throughout the cell cycle and the fission yeast Plo1 is responsible for bipolar spindle formation, the metaphase-anaphase transition and sister chromatid segregation, as well as septation (Glover et al., 1996; Ohkura et al., 1995), a search for interacting proteins that mediate Plo1 function was undertaken. The approach used here to screen a large number of proteins for interaction was the yeast two-hybrid method. This method has been used successfully for finding interactors of Plo1 with a bank of proteins produced during vegetative (mitotic) growth (Reynolds and Ohkura, 2003). In budding yeast, Cdc5 regulates meiotic function (Lee and Amon, 2003). No meiotic function for Plo1 in fission yeast has been defined yet. To find interacting proteins and to investigate the potential for Plo1 in meiotic functions, proteins produced in meiosis were examined for putative interaction by a yeast two-hybrid screen.

2.2. The Yeast Two-Hybrid Method

2.2.a. Outline of the two-hybrid system used

The yeast two-hybrid method uses a transcriptional activator that is comprised of two distinct functional domains. The DNA-binding domain recognises and binds a specific DNA sequence, while the activation domain interacts with transcriptional machinery to activate gene expression. Investigators have exploited these two domains by separating them, so that transcription can only occur if the two domains are brought into close proximity of one another. The yeast two-hybrid system used in this screen is based on the

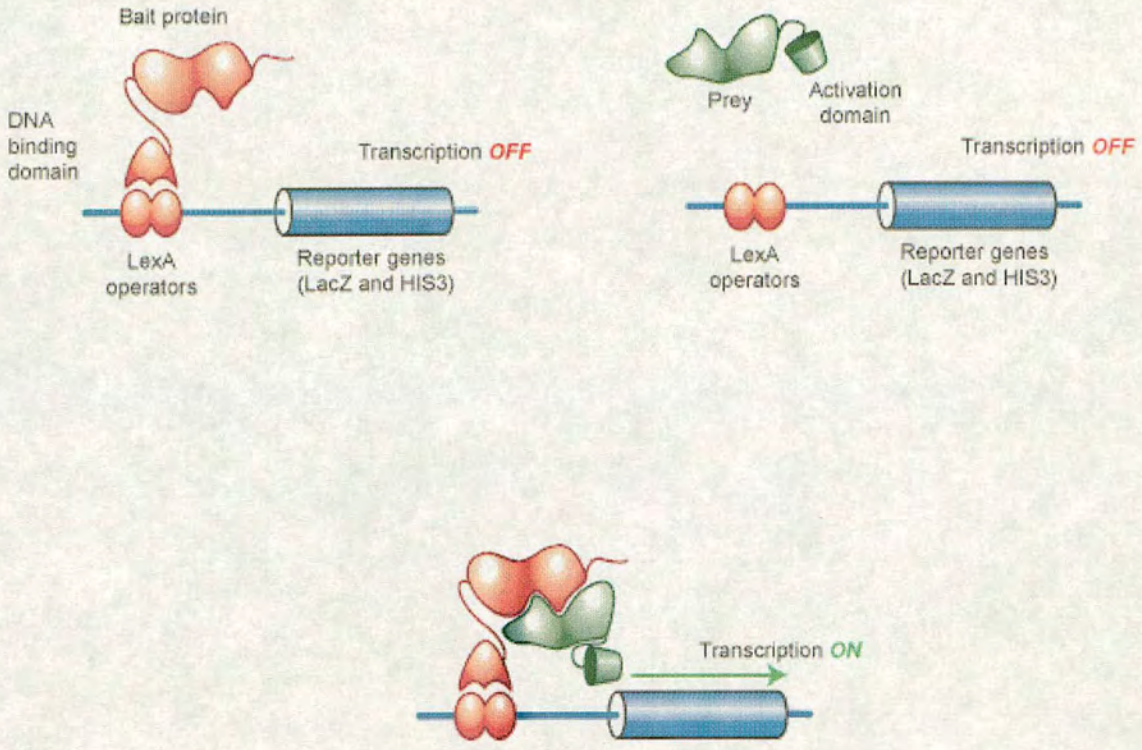

Figure 2.1. Diagram of the yeast two-hybrid system.
A) The Gal4 binding domain (BD) is continually in contact with the upstream activating sequence (UAS). If a prey protein does not interact with Plo1, the fused to the Gal4 activation domain (AD) does not come into close proximity with the UAS. Transcription of a reporter does not occur.
B) If a prey protein interacts with Plo1, the Gal4AD comes near to the upstream activating sequence, which in turn activates transcription of a chosen reporter.
(Adapted from http://www.dsbiotech.ch/site/technologies/yeast_two_hybrid)

Gal4 protein, which has been divided into the N-terminal DNA-binding domain and the C-terminal activating domain (Fields and Song, 1989). When the two are in contact with one another, they are able to activate transcription of any gene downstream of the upstream activating sequence (UAS).

In this study, full-length *S. pombe* Plo1 has been used to screen for interactors. Plo1 is referred to as bait and is fused to the DNA-binding domain of Gal4, so that it is continually in contact with the UAS. The library of Gal4 activation domain fusion proteins is encoded by cDNA and is referred to as prey. The cDNA library is discussed in section 2.3.a. If the prey protein interacts with bait (Gal4BD-Plo1), the fused Gal4 activation domain is brought near the UAS, stimulating transcription of the reporter gene (Figure 2.1).

2.2.b. Plasmids for the Plo1 screen

Full-length *plo1* was cloned into the plasmid, pGBT9, which contains the Gal4 binding domain and the auxotrophic marker *TRP1*. The prey plasmid contained cDNA, the Gal4 activation domain and *LEU2* as an auxotrophic marker. A pool of cDNA, produced from meiotic fission yeast cell mRNA, had previously been cloned into this plasmid using 5'-SalI and a 3'-NotI restriction sites by the Shimoda laboratory. The total number of original clones in the library was $1x10^6$. The proteins, because they were fused to the Gal4 activation domain, could be tested for interaction with Plo1 bait, and were.

2.2.c. Reporters in response to interaction and activation

Two reporters were used to assess interaction between bait and prey proteins. These are *HIS3*, encoding imidazoleglycerolphosphate dehydratase of budding yeast, an enzyme that is required in histidine biosynthesis and *lacZ* encoding β-galactosidase of *E. coli*. The

strain Y190, which is auxotrophic for leucine, tryptophan and histidine, was used for yeast two-hybrid assays. This strain has the two reporters, *HIS3* and *lacZ*, used to evaluate interaction. Upstream of each reporter is the UAS, which is activated when the Gal4 activation domain is within close proximity to this DNA sequence. If two proteins fused to the BD and AD physically interact, they will bring the BD and the AD close enough together to restore a functional transcription factor. Thus, the reporters are activated when an interaction occurs (Figure 2.1). Transcription of the reporter gene transcribed, *HIS3* (a nutritional selection marker) results in histidine prototrophy, and transcription of the *lacZ* gene results in blue coloration of yeast cells under an assay for *lacZ* activity.

The use of the *HIS3* reporter allows the investigator to adjust the sensitivity within the two-hybrid screen. The *HIS3* reporter can be transcriptionally activated easily and even weak protein-protein interactions allow production of sufficient His3 protein to produce His positive yeast. The experimenter can adjust the level of sensitivity by using an inhibitor of histidine production, 3-aminotriazole (3-AT). This tool lets the investigator decide the threshold for a signal to be detected, so that unwanted interactors are minimised.

2.3. Optimisation of the conditions of the screen

2.3.a. Amplification of the cDNA library

For yeast two-hybrid screening, a meiotic cDNA library was used in this instance to identify meiosis specific genes. The meiotic cDNA library (a gift of C. Shimoda) was made from fission yeast strain L968 wild-type h^{90}. This strain is capable of switching mating type from h^+ to h^- and vice versa. The strain was starved of nitrogen at 0 hours to induce meiosis and mRNA was purified from extraction time points of 2, 4, 6, 8, 10 and 12 hours. The

mRNA was pooled together and the first strand cDNA was synthesised, probably to be able to test a large number of putative interacting proteins at one trial.

The cDNA library was amplified by transforming supercompetent bacteria. A maximum of $1.07x10^6$ cDNAs was present after amplification of the cDNA library. The bacteria were collected and 1/10 was used to inoculate medium to purify plasmid DNA. The remaining amount was stored.

2.3.b. The first transformation of yeast with the meiotic cDNA library

Plasmid DNA containing the cDNA library was used for the two-hybrid screen by transforming yeast that already contained the bait plasmid (pGBT9 with *plo1* as insert, see section 2.2.b). In the first transformation, 700,000 cDNAs were tested for interaction with Plo1 in the first transformation.

Three concentrations of 3-AT, 10mM, 20mM and 30mM were used as a trial in the first transformation. The plates with 10mM 3-AT had approximately 500 colonies per plate. Some colonies grew larger after three days at 32°C. The large colonies were regarded as positives and were studied further, while the others were considered as background. At 20mM and 30mM 3-AT, approximately 500 transformants were found per plate also. At 30mM 3-AT, the difference between the size of large colonies and smaller background colonies was more pronounced.

In total from the first experiment, 111 of 700,000 prey proteins tested were positive for histidine production. At the various concentrations of 3-AT, the fractions of histidine

Table 2.1. Summary of three transformations, indicating fraction of histidine-positive yeast from cDNAs tested and the number of *lacZ* positive colonies.

Transformation	[3-AT] [1]	10 mM	20 mM	30 mM	40 mM	*lacZ* positive initial - final[2]
1st	111/700,000	27/200,000 [3]	40/300,000	44/200,000	—	24 - 14
2nd	151/1,200,000	—	—	—	151/1,200,000	13 -11
3rd	255/1,000,000	—	—	—	255/1,000,000	35 - 24

1) The fractions indicate the number of histidine-positive yeast out of the number tested.
2) The last column indicates initial and final *lacZ* positives. The final number represents those that are l*acZ* positive after single colony isolation. Initially, colonies were isolated to be positive for *lacZ* activity, but then were purified for single colonies, which were assayed again.
3) In the first transformation, 4 of 27 histidine-positive were *lacZ* positive at 10mM, 4 of 40 at 20mM and 6 of 44 at 30mM after single colony isolation.

positive colonies per number of prey proteins tested were 27/200,000 at 10mM 3-AT, 40/300,000 at 20mM and 44/200,000 at 30mM (Table 2.1. first row).

To diminish the probability of obtaining non-specific interactors, further selection was performed with a second reporter. His positive colonies were assayed for *lacZ* activity, by exposing the yeast to X-galactose (5-bromo-4-chloro-3-indolyl-β-D-galactoside). The β -galactosidase positive yeast have the capacity to oxidise the substrate X-galactose, resulting in an accumulation of a product that changes the colour of colonies from white to blue. Strains not expressing *lacZ* were white, while those that expressed *lacZ* were blue. This narrowed the pool of potential interactors to more specific interactors of Plo1. Out of 111 His positive yeast, 24 were positive for *lacZ* activity (Table 2.1, last column). After single colony isolation (described in section 2.4), 14 strains remained *lacZ* positive.

During the first experiment there was a high level of background when attempting to identify His positive yeast, with less background occurring under increasing amounts of 3-AT. To determine whether increasing the concentration of 3-AT resulted in any loss of interactors, before attempting to diminish the background in further experiments. The fraction of *lacZ* positive colonies out of His positive colonies were analysed at varying concentrations of 3-AT (Table 2.1). The percentage of *lacZ* positives of His positives was compared for the three concentrations of 3-AT. At 10mM 3-AT, four yeast strains were *lacZ* positive out of 27 His positive yeast (Table 2.1). Note this was after single colony isolation (described in section 2.4). At 20mM, four were *lacZ* positive out of 40 His positive yeast, and at 30mM, six of 44. The percentages at 10mM, 20mM and 30mM of *lacZ* positives to His positives are 14.8%, 10.0%, and 13.6% showing that about 10-15% remained after selection with *lacZ*, regardless of the increase in 3-AT concentration. Thus, by assessing the data from *lacZ* selection, the various concentrations of 3-AT did not

increase the selectivity significantly, suggesting that with the increase from 10mM to 30mM 3-AT, weaker interactors were probably not lost.

2.3.c. Increasing the selection stringency for histidine reporter activation

Since I had obtained a high level of background in the first transformation, and there was no loss of interactors with higher concentrations of 3-AT according to selection with *lacZ* during the first experiment, it seemed reasonable to increase the concentration of 3-AT to decrease background. A previously identified interactor was used as a positive control to determine the maximal concentration of 3-AT. The previously identified interactor, SPAC26H5.05 (Reynolds and Ohkura, 2003), was tested for growth under varying concentrations of 3-AT. The concentrations ranged from 0mM to 100mM, including 0mM, 10mM, 20mM, 30mM, 40mM, 50mM, 70mM and 100mM. SPAC26H5.05 formed medium-sized to large colonies at 10mM, 20mM, 30mM and 40mM, though slightly smaller colonies at 50mM. At 70mM the colonies were smaller and very few colonies exhibited proper growth on 100mM 3-AT. Thus, 40mM 3-AT indicated the highest concentration for a previously identified interactor of Plo1 to form viable colonies of a good size. In addition, since there was no decrease in the number of large His positive colonies at 40mM during the dose response experiment, I decided to use 40mM 3-AT in subsequent transformations.

2.3.d. Further transformations with the meiotic cDNA library

The second transformation resulted in less background using 40mM 3-AT and this concentration continued to be used for the third transformation. The number of cDNAs tested was calculated. In the second transformation, 1.2×10^6 cDNAs were tested and in the third, 1.0×10^6. 2.9×10^6 cDNAs were screened in total. From the histidine positive

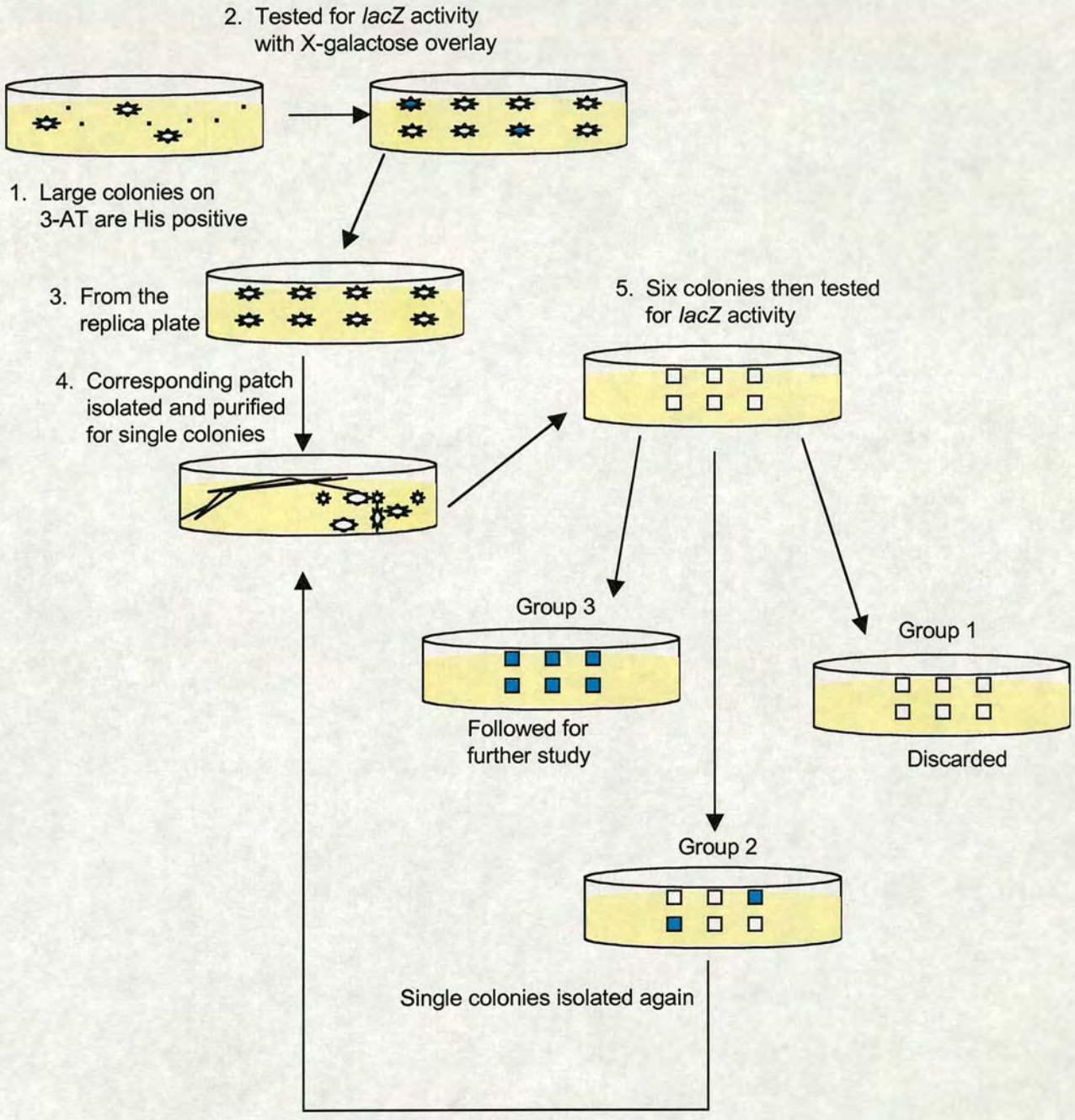

Figure 2.2. Single colony isolation.
Large colonies were chosen for further selection with *lacZ*, the second reporter. *lacZ* positive yeast were streaked for single colonies and six colonies were maintained. The six colonies were retested for *lacZ* actvity and three groups of yeast emerged. Blue boxes indicate *lacZ* positive patches of yeast, while white boxes indicate *lacZ* negative patches of yeast. Group 1 consisted of all six colonies as white or negative for *lacZ* activity. Group 2 had a mixture of activities and were purified for single colonies again, while group 3 were consistently positive for *lacZ* activity or blue and followed for further study.

colonies, the colonies that grew larger after three days at 32°C: 151 from the second transformation and 255 from the third transformation. These colonies were assayed for *lacZ* activity. From the second transformation, 11 of 151 colonies remained positive after single colony isolation and selection for His and *lacZ* (Table 2.1). This means that 7.3% remained positive comparing *lacZ* positives out of the pool of His positive colonies. For the third transformation, 24 of 255 remained positive, indicating 9.4%.

2.4. Retesting interaction after single colony purification

The aim of the next experiments was to confirm the prey as interactors after purifying for single colonies. One of the reasons for processing yeast colonies in this manner is because often, when the original colony is picked, there can be a mixture of yeast containing two different prey plasmids, or cross-contamination can occur between colonies. Yeast containing prey plasmids that were positive for *lacZ* activity were picked and streaked for single colonies. Six single colonies were maintained on the appropriate medium and were tested for *lacZ* activity (Figure 2.3). Three groups emerged. Group 1 had all six individual colonies negative for *lacZ* activity. Group 2 had a mixture of activities. For example, four of six colonies were positive and two were negative. Group 3 had all six colonies consistently positive for *lacZ* activity and interaction. Group 1 was discarded. Yeast from group 2 were purified again to obtain single colonies and retested using six colonies again. Yeast from group 3 were followed and used for further study as they were consistently blue with X-galactose assays (Figure 2.3). As they were confirmed as positive for *lacZ* activity, the prey within these yeast were considered as positive interacting-proteins.

The numbers of *lacZ* positive yeast before and after single colony purification are shown in the last column of Table 2.1. The majority (49 of 72), 68%, of putative interactors were confirmed as positive interactors of Plo1 after single colony isolation.

2.5. Preliminary identification of putative interactors

A method using PCR allows an investigator to identify gene products prior to extracting a plasmid from yeast (Figure 2.2). The yeast containing positive-interacting proteins were subjected to colony PCR. The cells were broken open under alkaline conditions at high temperature and a small amount of cell mixture was used as a template for PCR. The primers GADF and GADR were used and they correspond to sequences in the prey plasmid that flank the cDNA insert. cDNA was successfully amplified from all except PB11, PC2 and PD18.

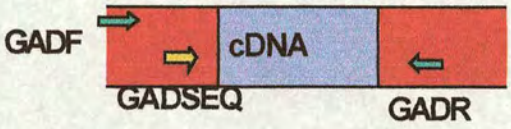

Figure 2.3. Colony PCR primers and identification by sequencing of PCR product. After cells from colonies were broken open under alkaline conditions, the template, consisting of plasmid Gal4 sequences in orange are and in purple cDNA insert, is amplified. The primers GADF and GADR were used to amplify a PCR product. Then the PCR product was sequenced using GADSEQ primer that lies downstream of GADF.

The GADSEQ primer used for sequencing was nested downstream of the forward primer (GADF), which was used for amplifying the product initially (Figure 2.3). GADSEQ primer corresponds to sequences within the vector and is common to each prey plasmid. The PCR products were treated with shrimp alkaline phosphatase (SAP), so that the dNTPs from the PCR mix would no longer be able to be incorporated into DNA. The

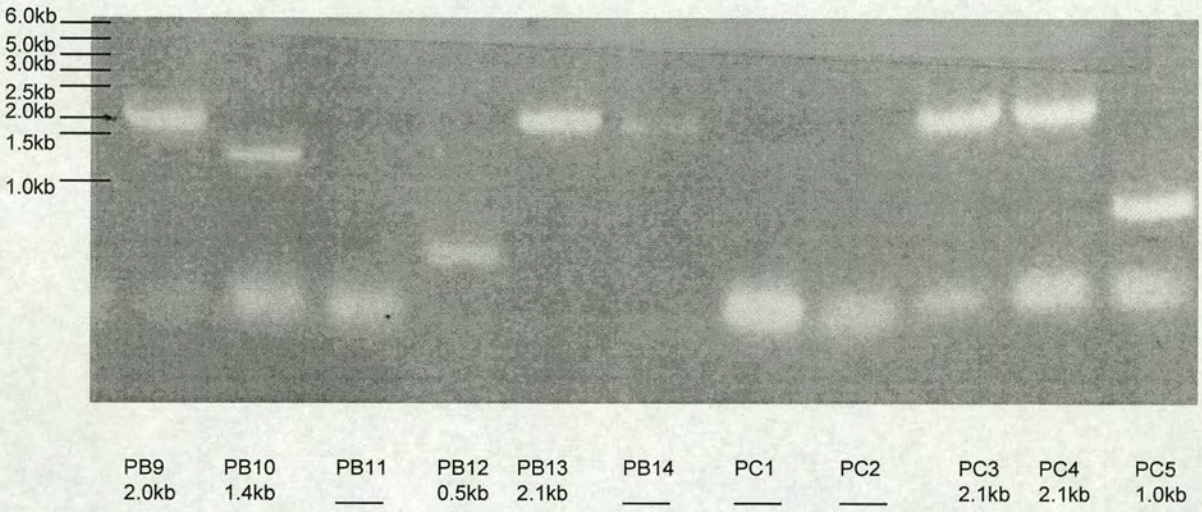

Figure 2.4. The sizes of colony PCR products are shown on an ethidium bromide-stained gel. Colony PCR products from *lacZ* positive colonies in the first and second transformations. The sizes indicate the approximate length of cDNA insert with the flanking sequences of vector.

excess primers were degraded by exonuclease (ExoI) treatment. The PCR product could then be sequenced without purification after heat inactivation of the SAP and ExoI enzymes.

The potential interactors identified in this screen are listed in Table 2.2 Of the 49 strains that were positive for interaction with Plo1, amplifying PCR product from seven strains was not successful. Three of the strains, that were not successfully used for PCR amplification, had plasmids rescued from them and identified as SPAC26H5.05. This left four strains not identified (PB11, PB14, PC2 and PC9). PCR products from two had no insert and upon sequencing were empty prey plasmid. One other strain had an unreadable sequence (PC7) from the amplified PCR product. Altogether, seven strains were discounted, four had no PCR product, two had no insert and one strain had unreadable sequence. For those strains that were identified by sequencing successful PCR product, *kms2* was found in 14 strains, SPAC26H5.05 was found eight times (including those times it was only identified from the plasmid), *sid4* was found three times and SPAC9.11 was found four times with two different transcripts (Table 2.2). *gpd3* was found in two different strains, accounting for 31 strains. Eleven strains had genes that were only identified once *kms1*, *sum2*, *htb1*, *ctt1*, SPAC4G9.07, SPBC1778.09, SPBC56F2, *sui2*, *pho2*, ribosomal protein S26 and *zym1* occurred once. So seven, 31 and 11 account for the 49 strains that were *lacZ* and His positive.

2.6. Rescue of prey plasmids

To confirm the interaction with Plo1 using appropriate controls, the next step was to isolate the prey plasmids. Colonies that had undergone single colony purification and had tested positive for *lacZ* activity were used for plasmid extraction. Notably, while attempting to rescue the prey plasmid from the first six interactors (PB1-PB6), only two out of the six

Table 2.2. Preliminary identification of PCR products from *lacZ* positive yeast.

Strain Name	Original Name	Strength of Interaction[2]	PCR Product Size	Gene Name Identified by Sequence
PB1	20-1	Strong	1.6kb	SPAC9.11
PB2	10-8	Medium	1.7kb	SPAC9.11
PB3	10-26	Medium	2.0kb	*kms2*
PB4	20-38	Strong	1.8kb	*sum2*
PB5	20-40	Medium	2.0kb	*kms2*
PB6	30-44	Medium	2.0kb	*kms1*
PB7	10-17	Strong	2.2kb	*sid4*
PB8	30-6	Medium	4.5kb	SPAC26H5.05
PB9	10-4	Weak	2.0kb	*kms2*
PB10	30-17	Weak	1.4kb	*htb1*
PB11	30-30	Weak	N/S*	
PB12	10-27	Weak	0.5kb	*ctt1*
PB13	20-25	Weak	2.1kb	*kms2*
PB14	30-42	Weak	N/S*	
PC1	17	Strong	2.0, 4.5kb	*gpd3*
PC2	22	Strong	N/S*	
PC3	31	Weak	2.1kb	*kms2*
PC4	48	Weak	2.1kb	*kms2*
PC5	58	Weak	1.0kb	SPAC4G9.07
PC6	65	Weak	1.0, 1.4kb	SPBC1778.09
PC7	84	Strong	0.7kb	unreadable sequence
PC8	101	Weak	1.0kb	*sui2*
PC9	113	Weak	N/S*	
PC10	128	Weak	0.5kb	SPBC56F2 non-coding[1]
PC11	136	Weak	1.0kb	*pho2*
PD1	8	Strong	2.1kb	*kms2*
PD2	9	Strong	2.5kb	*sid4*
PD3	21	Medium	1.0, 2.0kb	*kms2*
PD4	40	Weak	0.5kb	*gpd3*
PD5	48	Strong	1.0, 2.0kb	*kms2*
PD6	53	Strong	2.0, 4.5kb	SPAC26H5.05
PD7	61	Medium	2.5kb	*kms2*
PD8	101	Medium	2.0, 4.5kb	SPAC26H5.05
PD9	126	Medium	2.0kb	SPAC9.11
PD10	129	Medium	2.0, 4.5kb	SPAC26H5.05
PD11	136	Medium	2.0, 4.5kb	SPAC26H5.05
PD12	154	Medium	2.4kb	*kms2*
PD13	170	Medium	2.5kb	*sid4*
PD14	182	Medium	1.8, 3kb	SPAC9.11
PD15	196	Medium	N/S*	SPAC26H5.05 from plasmid
PD16	203	Medium	N/S*	SPAC26H5.05 from plasmid
PD17	223	Medium	1.0,1.5kb	*kms2*
PD18	235	Medium	N/S*	SPAC26H5.05 from plasmid
PD19	255	Weak	0.9kb	ribosomal protein S26
PD20	38	Weak	no insert	empty prey
PD21	77	Weak	no insert	empty prey
PD22	127	Medium	3.0kb	*kms2*
PD23	233	Medium	0.7kb	*zym1*
PD24	241	Medium	3.0kb	*kms2*

*N/S: the colony PCR was not successful.

1. Strain PC10 had an amplified PCR product that shared sequence identity with the non-coding region of the gene that encoded SPBC56F2 protein. 2. Strength of interaction assayed by *lacZ* activity in strong, medium or weak blue colour.

interactors gave at least one prey plasmid out of seven to 20 plasmids isolated from each strain, while the other four interactors gave only bait plasmids out of 20 plasmids isolated.

The propensity for the bait plasmid to be recovered more often than the prey plasmid may be due to the size of the plasmids. The bait plasmid was 5.5kb and the prey plasmid was 6.6kb without insert. When plasmids are extracted, the smaller bait plasmid is probably more easily rescued. Thus, a more extensive protocol for losing bait was devised and used. Yeast were grown in liquid medium with tryptophan to promote more doublings in an effort to lose the bait plasmid. The yeast were plated onto solid medium containing tryptophan and grown for 3-5 days at 32°C. Then, after replica plating from medium with tryptophan onto plates without tryptophan, some colonies died without tryptophan. The colonies that did not survive without tryptophan had lost the bait plasmid carrying *TRP1*. They were used for isolation of prey plasmid.

The prey plasmids were rescued by extracting them from yeast, transforming bacteria and recovering the plasmids. The rescued plasmid was analysed by gel electrophoresis and a restriction digest with HindIII was performed to differentiate between bait and prey plasmids (Figure 2.6). The bait plasmid containing *plo1* cDNA resulted in a restriction pattern of 4.6kb, 1.5kb, 972bp and 476bp (Figure 2.6). The prey plasmid would have 6.0kb fragment and 695bp plus the size of the cDNA insert and subtracted any fragments resulting from digestion of the individual cDNA.

Each prey plasmid was digested with SalI and NotI to release the insert of cDNA (Table 2.3). By comparing the size of the released fragment with the size of colony PCR product, the plasmid extracted was correlated with the cDNA contained within a yeast strain. In general, the sizes of insert released from a SalI/NotI digest were seen as 100-200bp smaller than that of the PCR product. In larger-sized DNA fragments, it is difficult to distinguish

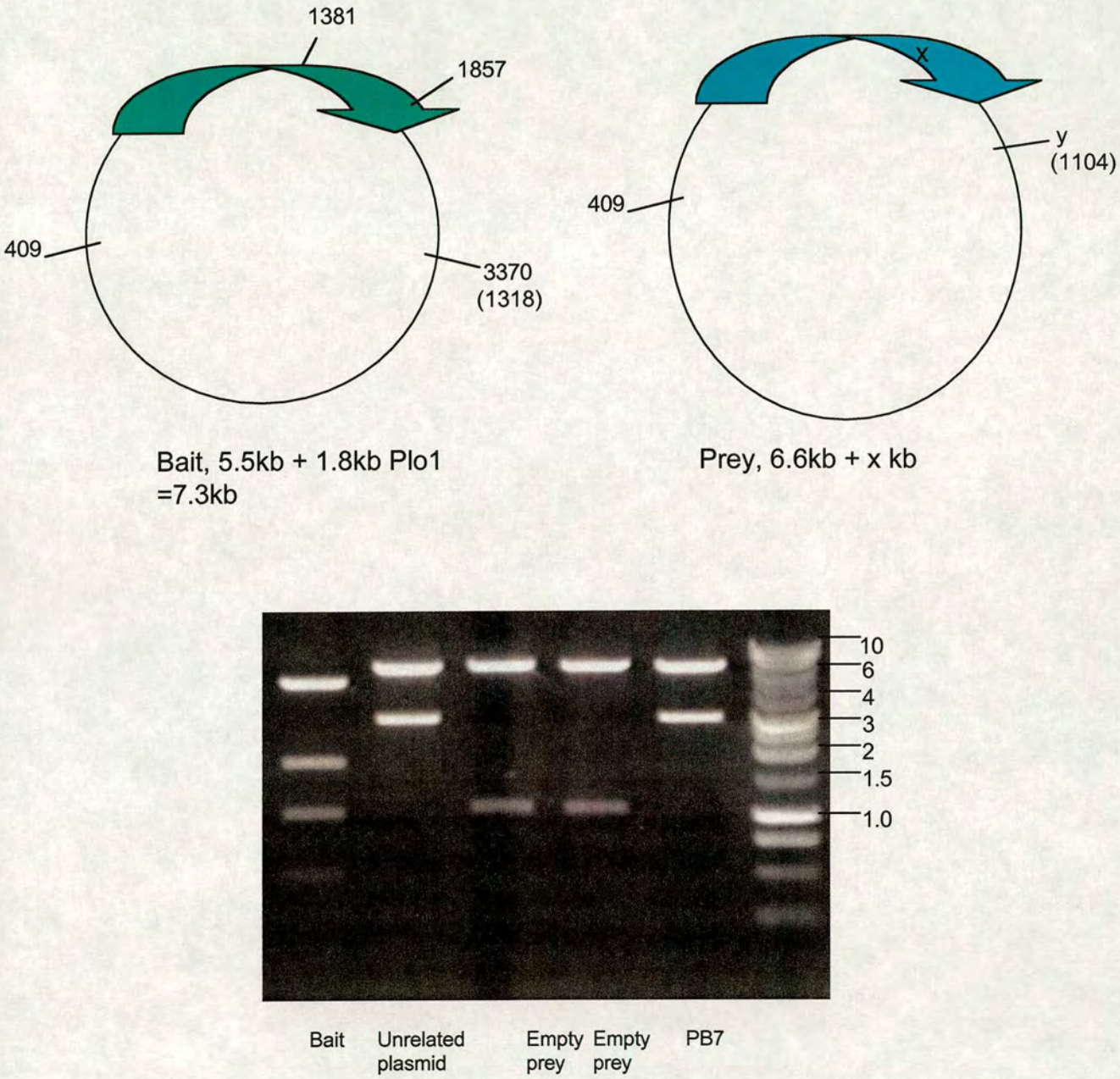

Figure 2.5. Differentiating between bait and prey plasmid after plasmid recovery.
The gel shows the two yeast two-hybrid plasmids and the different expected sizes from each after HindIII digestion. The bait plasmid results in four fragments of 4.6kb, 1.5kb, 972bp and 695bp, whereas the prey would vary depending on the number of HindIII sites within the cDNA insert. The difference in the largest fragment, 4.6kb in bait and 6.0kb in the prey aids to distinguish between bait and prey, in addition to the pattern of restriction digest.

between 0.1kb and 0.2kb (100-200bp). The PCR product extended outwith the cDNA for a total of 135 base pairs of PCR product in excess of size compared with a restriction-digested fragment. Thus, the SalI/NotI restriction digests of plasmids carrying as DNA of a positive interactor, which are 100-200bp smaller than the PCR product correspond to that given PCR product.

The rescued plasmids were then sequenced with GADSEQ primer. Three plasmids and their corresponding colony PCR products were not sequenced successfully. After sequencing the remaining plasmids, the basic local alignment search tool at www.ncbi.nih.gov/BLAST was used to determine the identity of the cDNAs contained in the rescued plasmids. I compared the identity of plasmid sequences with the identity of PCR products (Table 2.2), confirming 39 of 42 PCR products identified. PC6 and PC8 had different sequence identities for PCR products and plasmids, for which the reason is unclear (Table 2.4). PD17 PCR product and plasmid sequences were not in agreement, probably because the plasmid had rearranged (Table 2.3. see * footnote).

2.7. Distinguishing between true and false positives

To distinguish between true and false interactors, the rescued plasmids were then used to retransform yeast with either empty bait vector or bait plasmid containing Plo1. True interactors are those prey plasmids that are positive for interaction, when transformed with Plo1-containing bait plasmid. Meanwhile, the same prey in combination with empty bait vector would be negative for interaction. True interactors include SPAC9.11, *kms2*, *kms1*, *sum1*, *sid4*, *htb1*, *ctt1*, SPAC26H5.05 and *zym1* (Table 2.3). False positive interactors are those that with empty bait vector remain positive for interaction. *gpd3* was a false positive, as the prey self-activated reporter genes (Table 2.4). The other interactors exhibited far too weak *lacZ* activity were not examined further (Table 2.4).

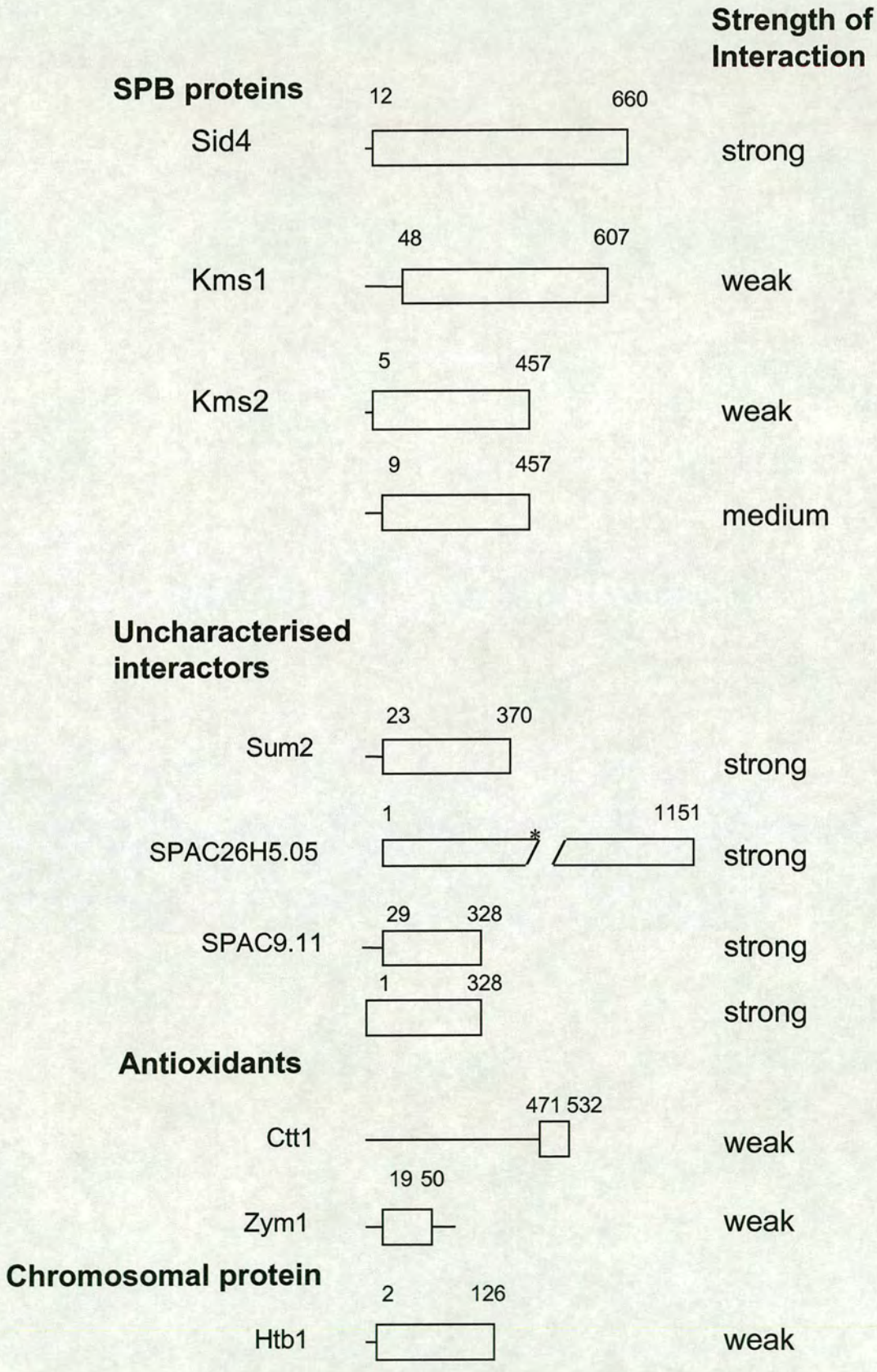

Figure 2.6. Depiction of the Plo1-interacting proteins.
The boxes represent the proteins, and the numbers indicate the amino acids encoded by cDNAs that were isolated as true interactors.
*/ / This is a full-length protein, but is broken up here because of its length.

Table 2.3. True interactors that were positive with Plo1 and negative with empty bait for *lacZ* activity.

Identity of Sequence [number of amino acids]	cDNA starting amino acid	PCR product size (kb)	Sal1/Not1 digest Size of insert (kb)
SPAC9.11 [328]	1- 29-	1.7 1.6	1.5 1.4
Kms2 [457]	5- 9-	2.0 2.0	1.9 1.9
Kms1 [607]	48-	2.0	2.1
Sum2 [370]	23-	2.0	1.8
Sid4 [660]	12-	2.5	2.2
Htb1 [126]	2-	1.4	1.4
Ctt1[532]	471-	0.5	0.5
SPAC26H5.05 [1151]	1-	2.0, 4.5	4.5
Zym1 [50]	1-	0.7	0.5
Unidentified *(PD17)		1.0, 1.5	5.0

* Plasmid PD17 was sequenced, but continually had a mix of sequences and was most likely a dimerised, rearranged plasmid. The size of the PCR products and released fragments from SalI/NotI digestion are shown here, which are not consistent. The colony PCR product was sequenced as *kms1*, but the plasmid was very difficult to sequence with two peaks at most positions and two different identifications being made.

Table 2.4. False interactors that were positive with empty bait for *lacZ* activity, or far too weak for interaction.

Plasmid number	PCR product sequence	Plasmid sequence
PB11	N/S	N/S
PC6	SPBC1778.09	*vip1*
PC8	*sui2*	*sui1*
PC10	SPBC56F2 non-coding region	SPBC56F2
PC2	N/S	N/S
PC7	N/S	unreadable sequence
PC9	N/S	N/S
PB14	*pap1*	*pap1*
PC5	SPAC4G9.07	SPAC4G9.07
PC11	*pho2*	*pho2*
PD19	ribosomal protein S26	ribosomal protein S26
PD20	no insert	no insert
PD21	no insert	no insert
PD4*	*gpd3*	*gpd3*

*-*gpd3* self-activates, meaning that it causes the reporters to be activated even when the bait vector is empty. The rest of the plasmids have far too weak interaction with Plo1. N/S means that the sequencing was not successful.

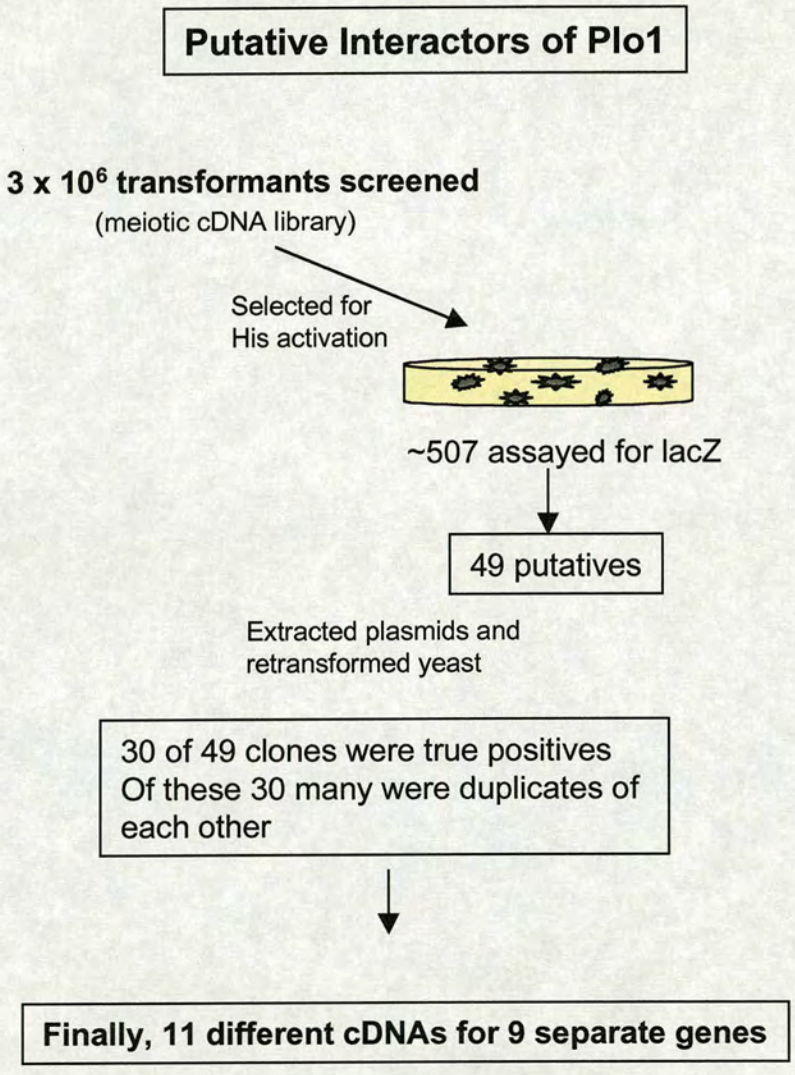

Figure 2.7. Diagram of the number of cDNAs tested and the process of selection to obtain true interactors. Narrowing down the specific interactors of Plo1 from the maximum number of cDNAs expressed from the library to the specific plasmids from strains reintroduced into yeast.

5′ pGAD424
c ccg ggg atc cgt cga c*cc acg cgt ccg*

kms2 (aa5-) PB9 — atc cct ttt gac gat
I P F D D

kms2 (aa9-) PB5 — gac gat att gtg cgc
D D I V R

kms1 (aa39-) PB6 — ccc gct tct gca cca
P A S A P

sid4 (aa1-) PB7 — atg gat gag gct ttt
M D E A F

sum2 (aa23-) PB4 — cgg acg cgt ggg ttg
L..

SPAC9.11 (aa29-) PB1 — gaa atg gag tat ccg
E M E Y P

SPAC9.11 PB2(aa1-) — cgg acg cgt ggg TAG

ttc cac TAA tcg cac ctt ata aac aaa ttg aat ctt tac tcg
aat aaa caa gct aaa gtt gtg aaa agc gaa gtg aac ttt tca
ctg ttt caa gat aat cac gtt gac cta ctt TAG gaa gtt ttt
aaa tac act aag aat tgc aga ttt tcc TAG ttg gta ctt ttg
tgt ttt ttg tca aat cct tat gac act tgt tca acc ctt ttt
ttc ttt att ata TAA atg
M (start codon)

Figure 2.8. Reading frame of the cDNA encoding each interactor.
The sequences that were found to be in-frame transcripts. gt cga c corresponds to the 5'-SalI site. The sequences in italics are a linker in the library which would have been used in the forward primer to make the cDNA library. The sequences on the right are those of the various in-frame transcripts of Plo1-interacting proteins. SPAC9.11 (PB2) has five stop codons (capitalised) in-frame prior to the start codon methionine.

In total, thirty true interactors remained out of the forty-nine after single colony isolation. However, several of these thirty were duplicates of the same clone. Nine proteins were found to interact with Plo1 finally (Figure 2.7): Sid4, Kms1, Kms2, Sum2, SPAC26H5.05, SPAC9.11, Ctt1, Zym1 and Htb1. Two cDNA transcripts of differing length encoded SPAC9.11. There were two transcripts encoding Kms2 protein also. The other seven proteins were represented by a single transcript. Thus, eleven cDNAs encoding nine proteins were identified as true interactors (Figure 2.8).

2.8. The reading frame of the transcripts

cDNAs of *sid4*, *kms1*, both transcripts of *kms2*, *sum2* and both transcripts of SPAC9.11were in-frame. However, within SPAC9.11 there were four stop codons present upstream of the Gal4AD in the longer transcript. Thus, SPAC9.11 was no longer considered. The other four cDNAs encoding putative Plo1-interacting proteins, include *htb1*, *zym1*, *ctt1* and SPAC26H5.05, were not in-frame (Figure 2.9). Frameshift surveillance systems can suppress out-of-frame mutations to produce the correct protein (Strauss, 1999), meaning that an out-of-frame transcript in a two-hybrid assay could be relevant. However, *htb1* encodes a histone, *zym1* a metallothionein, *ctt1*, catalase. These abundant proteins may have been present in a high copy number and have characteristics that allow non-specific interaction. SPAC26H5.05 is an unknown ankyrin-repeat protein and was identified previously in this laboratory where amino acids 533-1151 were positive for interaction (Reynolds and Ohkura, 2003). Whether the plasmid (pNR 461 laboratory plasmid) contained cDNA in-frame with the Gal4AD is not known at this time. Further to this, one would need to determine if the interaction occurred within fission yeast by immunoprecipitation and also find a functional and biological reason for the interaction by assaying localisation and genetic interactions. At this point these four proteins that were not in-frame with the Gal4AD were no longer considered. Although both transcripts of SPAC9.11 were in-frame, it is not clear how the longer transcript produced a positive-

interacting protein considering there are five stop codons between the Gal4AD sequence and SPAC9.11 cDNA.

2.9. Characteristics of positive interactors

The in-frame true positive Plo1-interacting proteins Sid4, Kms1, Kms2 and Sum2 were studied further. Information from these candidates is important to determine relevance. Biological information, particularly if any function is known, the localisation and information from genetic experiments or gene expression studies was first gathered. Then, using alignments to search for homologues in other organisms and structural properties determined from protein sequence data were considered. Recently, a binding motif and a potential phosphorylation consensus sequences of human Plk1 have been identified. For Sid4, Kms1, Kms2 and Sum2, putative binding and phosphorylation sites were examined within protein sequences.

Thus, for each protein the following list of characteristics were considered:

Biological Information	1) Function if known
	2) Localisation
	3) Information from genetic studies
	4) Gene expression during meiosis
Structural information	5) Homology
	6) Structural properties, search for domains
	7) Putative binding motifs
	8) Potential phosphorylation consensus sequences

The biological information came from previous published studies and from gene expression studies performed by the Sanger Centre, which use microarray data (Mata and Bahler,

2003). For structural information, the basic linear alignment search tool (BLAST) program (Altschul et al., 1990) was used in conjunction with a non-redundant database to identify homologues from other organisms of the fission yeast proteins (http://npsa-pbil.ibcp.fr, the website of Pole Bio-Informatique Lyonnais, Network Protein Sequence Analysis). The recommended threshold of $1xe^{-06}$ was used, which is approximately 1/500 that the sequence would be identified as a homologue randomly. The e-value indicates the probability that the sequence identified was identified by chance, not by the amino acids shared in common reflecting relatedness. Homology was assessed and then, various programs were used to analyse protein sequence to identify structural motifs or functional domains defined by conserved sequences. Lastly, the Plk binding and phosphorylation sites were searched for.

2.9.a. Sid4

The fission yeast protein Sid4 is essential for cell survival. Sid4 functions in septation and it localises to the SPB throughout the cell cycle (Chang and Gould, 2000). Sid4 is part of the septation initiation network (SIN) and it is required for all components of the SIN to localise to the SPB (Chang and Gould, 2000; Krapp et al., 2001). Without Sid4, none of the components of the SIN can localise and septation does not occur, leading to elongated, multinucleate cells. However, Sid4 does not interact directly with components of the SIN. Instead, it interacts with the docking protein Cdc11 (Krapp et al., 2001). In addition, Sid4 forms homodimers (Chang and Gould, 2000; Tomlin et al., 2002).

From a random mutagenesis screen, high *plo1*$^{+}$-dependent mutants were identified as components of the septation initiation network. Mutations were allelic to *cdc11*, *cdc7*, *cdc15*, *spg1* and *sid2*. These mutants depended on high levels of Plo1 for cell survival

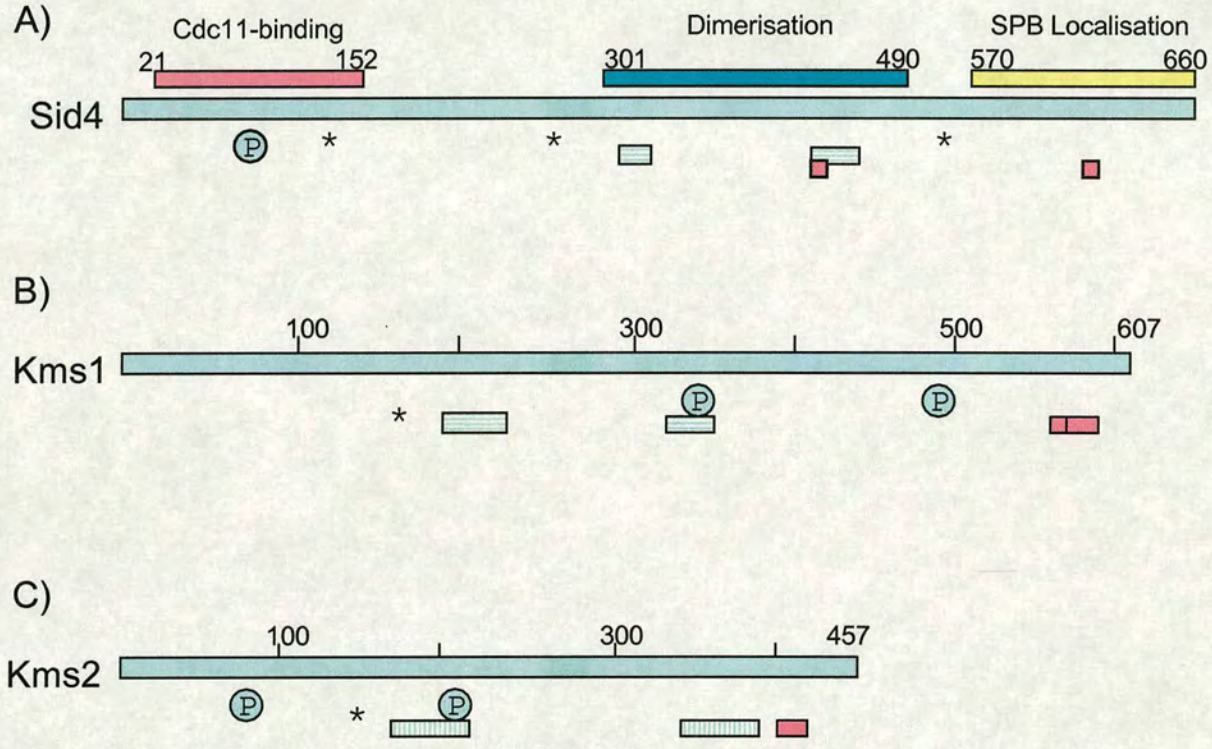

Figure 2.9. A) Depicts the different known domains of Sid4. Above boxes indicate functional domains, such as Cdc11-binding at amino acids 21-152, a region for dimerisation at 301-490, and a SPB localisation domain at 570-660. Below, striped boxes indicate coiled-coil domains at 334-354 and 434-461,the pink boxes represent putative transmembrane domains.Circled P's represent putative phosphorylation sites. Asterisks represent potential binding sites. B) Depicts Kms1 and C), Kms2.

(Cullen et al., 2000). Therefore, Plo1 acts in the SIN pathway. Sid4 functions at the top of the SIN (Chang and Gould, 2000).

From the known biological function of Sid4, septation, and from genetic information that high levels of Plo1 protein sustain mutants of the SIN pathway (described in section 1.12.d), Sid4 is a highly relevant Plo1 interacting protein. Plo1 drives septation when overexpressed (Ohkura, 1995) and this may be mediated by interaction with Sid4. In addition, Sid4 is a SPB protein and Plo1 localises to the SPB for part of the cell cycle. Plo1 localisation to the SPB is associated with septation (McCollum and Gould, 2001; Mulvihill et al., 1999; Tanaka et al., 2001).

Information from fission yeast microarray data from gene expression studies is available on the Sanger centre website. These data were collected from two strains of yeast undergoing meiosis, one a control wild-type and another called *pat1-114*. Yeast were grown in liquid culture at 25°C for 14 hours, then the wild-type strain was starved of nitrogen and incubated at 34°C, while the *pat1-114*strain was starved of nitrogen and grown at 30°C. The RNA was extracted and PCR products of 99.3% of the known fission yeast genes were spotted on microtitre plates. The data for gene expression of all mRNA levels of known fission yeast genes tested during sexual differentiation. The data is available at www.sanger.ac.uk/S_pombe (Mata and Bahler, 2003). According to mRNA expression profiles, *sid4* expression is not increased or decreased during meiosis. *sid4* is expressed during mitotic growth, at time point 0h of sexual differentiation, meaning that it is expressed during mitosis. Nitrogen starvation induces meiosis and the time of nitrogen starvation is considered timepoint 0hours, where *sid4* is expressed. *sid4* expression is not altered when meiosis is induced. S phase occurs at approximately 2 hours, the meiotic division between five and seven hours and sporulation takes place at 10 hours.

Using BLAST alignments to seek out homologues of Sid4 did not reveal any homologues by sequence similarity.

Through analysing Sid4 protein sequence, structural motifs and functional domains were searched for. Coiled-coils were predicted. They are found in a wide variety of proteins. Structurally they are composed of two alpha helices that wind around each other. The predicted parallel two-stranded coiled-coils are thought to play a role in protein-protein interactions. Seven amino acids make up two turns within a helix and predictions for coiled-coils take into account every seventh amino acid. If each seventh amino acid is hydrophobic, one face of that helix is hydrophobic and can easily be packed next to another helix that has one hydrophobic face(Branden and Tooze, 1999). Eventually the two helices coil and intertwine around each other. From sequence analysis, coiled-coils were predicted at amino acids 334-354 and at 434-461 of Sid4 (Figure 2.9.A) using the program at www.ch.embnet.org/software/COILS_form.html (Lupas et al., 1991). The coiled-coils may be important for structural folds of Sid4 or for facilitating protein-protein interaction.

For transmembrane domain predictions, the DAS program at http://www.sbc.su.se/~miklos/DAS/maindas.html was used (Cserzo et al., 1997). The program predicted transmembrane domains portrayed as pink boxes in Figure 2.9.A. The second potential transmembrane domain corresponds to the C-terminal region of amino acids 570-660, which are required and sufficient for SPB localisation (Niwa et al., 2001; Tomlin et al., 2002) (Figure 2.9.A). The minimal domain for dimerisation is found at amino acids 301-490 (Figure 2.9.A) (Tomlin et al., 2002) and the domain for Cdc11 interaction exists at amino acids 21-152 (Figure 2.9.A) (Tomlin et al., 2002).

In searching for putative binding sites and phosphorylation consensus sequences within Sid4, one potential binding site and one putative phosphorylation site were found.

Sid4, one potential binding site and one putative phosphorylation site were found.

Plks bind phosphorylated serines and threonines through their C-terminal region. The phosphorylated serines and threonines are bound depending on the amino acids surrounding the serine or threonine residues. The optimal binding motif is MGSpTPL (Elia et al., 2003). Plk1 can bind MGSpSPL, but at $1/7^{th}$ of the affinity (Elia et al., 2003). There is a strong preference for serine to be at the –1 position and a slight preference for proline at +1, given that the phosphorylated amino acid is referred to as position 0. Therefore, the binding motif is S-pS/pT-P/X, where / means or and X is any amino acid. For Sid4, there are several potential sites for binding. No SpSP sites exist, but SpTP do at amino acids 135, 272 and 533 and are marked as asterisks in Figure 2.9.

Phosphorylation consensus sequences for Plo1 have not been elucidated, but for human polo-like kinase Plk1, D/E-X-pS/pT-F/X is the consensus sequence, where X is any amino acid, except cysteine. There is a strong preference for a hydrophobic residue at the second X position (Nakajima et al., 2003). Sid4 has one putative phosphorylation site. The consensus sequence is present in ENpTKKE (T79, threonine is at amino acid position 79) (represented by a circled P in Figure 2.9.A).

2.9.b. Kms1

Kms1 (karyogamy and meiotic segregation) is required for the integrity of the spindle pole body during meiosis, but null mutants do not exhibit any defects under vegetative growth conditions. Disruption of the *kms1* gene leads to disintegration of the SPB and destruction of telomere-led bouquet formation (Niwa et al., 2001; Shimanuki et al., 1997). The chromosomes are attached to the SPB at the telomeres, rather than the centromeres in

```
Kms1 MLNERDFDLIFDSYDFKHEGKVHLSNFLPIINDLQLLHPASAPPLLSEFQKQCTLEFVRQNADLSI 66
Kms2 MDEYIPFDDIVRQYDPDYTGKVSIQAFLEIVDDVDALRLNPEAPLLDNEQRQSAQDFIKDNSEIVV 66
Cons * :   ** *. .** .: *** :. ** *::*:: *:  . .***.: *:*.: :*:::*::: :

Kms1 TKDNFRDVYKKLTENEDDSFANQAEKPSMEQQNSKNSIKEDANEHSVNSAHSKSSSNASPESLNPS 132
Kms2 STSEIKNLFYELTGLDPDTL------------------------PVNKLALRENGVLPRKSVAKP 107
Cons :..:::::: :**  : *::                          .**.   :...  . :*:  .

Kms1 QMMSKRLSLPPMSQFTDSDFVNILR-TPFAQSTPLN----RNTSSRNTEMPLVRKDKPDFSNGHHD 193
Kms2 QKISENRIKRKDMFYQDASYITPRKGSPLSHSTPLSMFRTKNEYGSNKGFSHINKENADDS----- 168
Cons * :*:.        : *:.::.  : :*:::****.    :*  . *. :. :.*::.* *

Kms1 LIKQITELQDMLDKARDQARKKSRTVDILEGKVNELTHQLNMAD----SKYNESKVANNSQNNQIK 255
Kms2 ---LIQQLYERIELQAAELRSKDEQVKELNARNAKLLEELDSSEEACKSCYTQAKTWEKKFREALR 231
Cons     * :* : ::    : *.*.. *. *:.:  :* .:*: ::    * *.::*. ::. .: ::

Kms1 TLK---AQNLNIHKNFQKIQSELIQTNSGLYSTKKELSALQVRYATLLRKFTDQTKKIEELSLAAS 317
Kms2 DSKEYAAQLQTIHEEYEQQQAHIVRMEELIHAVEKE-----------RKTETDYMKKESLSEQKE 285
Cons   *   **  .**:::::: *:.::: :. :::.:**            **   :  * *.**   .

Kms1 RSSENE-NTIRRLALENHELKNSNNQLNNHIDDLTREKHLIALSNNPKGDEFLSPSNLDEMVYSKE 383
Kms2 RGAFMESNMILEEKVAHLQLENEQLRL------FFKEKAPQPFQNHPPYQ--------------- 329
Cons *.:  * * * .  : : :*:*.: :*      : :**   .:.*:*  :

Kms1 VGLSFTQPSVCISIPAVGMRESEELRELEFKCKQQKKTIEECKHISQSLQSSLTAESSRNKELVAG 449
Kms2 -NLKITFPSPFFHIPYIP---------------------------------KTETLNDSQFAAG 359
Cons  .*.:* **  : ** :                                   .:*: .:.::.**

Kms1 FLMLSEEIGIQKWIIQSLSKMSPTLNDFCRRYDSSMPTYEESSHECTVLSSFSDDETGLMATNTTM 515
Kms2 LSLLASELESQKNLLKKFENLK---------------------------------------- 381
Cons : :*:.*:  ** :::.:.::.

Kms1 NNSSKDFMASQDTVNADNPHFLATKGQPLLLLSVMKSNILRLFYVLFLFACFYGLDYILCAELLQA 581
Kms2 KKSSKDFLSPSTILS----------NAFSKVSLPNSLILRVLFFSLLFIIGIHIFYFLFF----- 431
Cons ::*****::..  :.          :.:  :*: :* ***:::. :**     : *:*

Kms1 FLRVVFTFCEHIIILLYGRYELVQPS---------- 607
Kms2 ----------HVATIQQWPFLFWLPSTKFDNRWSPT 457
Cons          *:  :    : :  **
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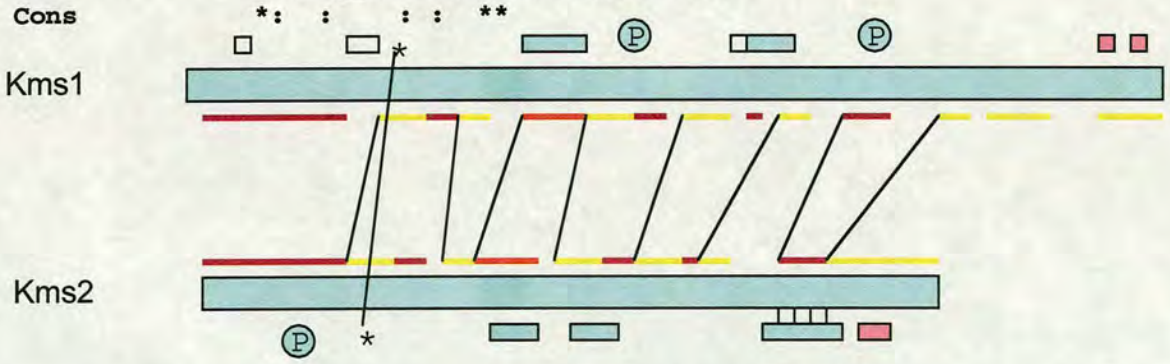


Figure 2.10. Alignment between Kms1 and Kms2. *Asterisks in the alignment represent where amino acids are identical and the dots are where amino acids may be similar in hydrophobicity or charge. The underlined letters represent amino acids that correspond to binding domains or putative phosphorylation sites. The STP sites in the third row are conserved.
The lower diagrammatic representation of Kms1 and Kms2 show where conserved areas are. The red line indicates a high degree of homology, the orange, very good homology and yellow, good homology. The black lines show to which coloured lines the areas of homology are.

meiotic prophase, forming a bouquet-like structure, and Kms1 itself localises to the SPB throughout the cell cycle. *kms1* is synthetically lethal with *kms2* (Shimanuki et al., 2002).

Although Kms1 functions in meiosis, *kms1* is not a meiosis-specific gene. It is expressed at time point 0h of sexual differentiation experiments, prior to meiosis. During meiosis, profiles of gene expression show that *kms1* mRNA is upregulated and particularly at induction of meiosis and S phase, then it dips at metaphase I and rises back at metaphase II of meiosis (Figure 2.10 top panel).

Using BLAST searches, I found that ZipA protein from *Dictyostelium discoideum* scored well in alignments with a value of $9e^{-07}$. Other proteins that were identified during BLAST searches fell below the threshold of $1e^{-06}$, but did include Kms2 at $6e^{-04}$, among other s (a putative centromeres protein in rice Q7XEH4 ($4e^{-06}$), human early endosome antigen Q15075 ($2e^{-04}$) and *Dictyostelium* myosin heavy chain Q869R0 ($5e^{-04}$). ZipA scored very high for coiled-coils throughout the protein, so the protein sequence of Kms1 lacking the coiled-coil domains (see next paragraph) was used to identify homologues. In this case, ZipA and the other mentioned proteins were not found as homologues. Kms2 did surface as the closest related protein with a low value of 0.15 when Kms1 without its coiled-coil domain was used for BLAST searches.

Kms1 has coiled-coils at amino acids 191-239 and 327-354 (Figure 2.9.B). Two EF-hands were found at amino acids 5-76 and at amino acids 556-602 (www.sanger.ac.uk). An EF-hand is a tertiary structure where two helices are connected by a short calcium-binding loop. Upon binding calcium, the EF-hand conformation changes. Kms1 also has

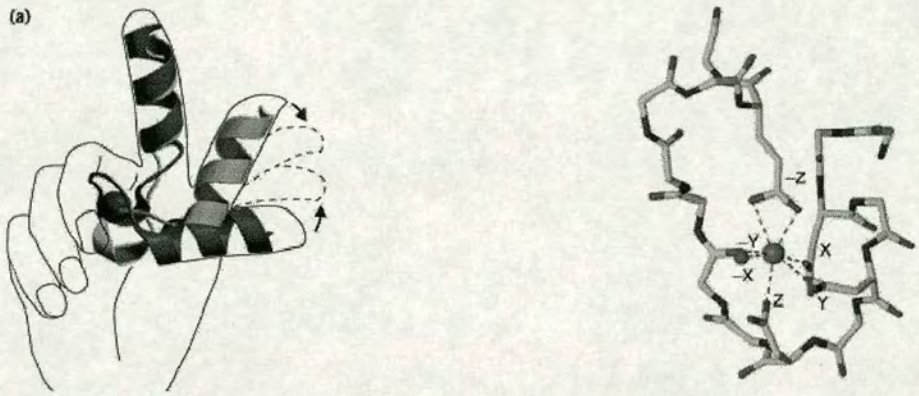


Figure 2.11. Left. An EF-hand has helix E traversing down the index finger and helix F at an angle winding up the thumb. Right. Calcium binds in the cleft of the two helices (Lewit-Bentley and Rety, 2000). Figure 2.12. EF-hand. Helix E goes down the index finger and Helix F goes up the thumb. When calcium binds, Helix F moves away from the closed position into the open position (Kretsinger and Nockolds, 1973).

transmembrane domains at amino acids 553-575 an 582-604 that are probably important for the protein to be anchored to the spindle pole body.

There is one potential Plk binding site S-pS/pT-P/X in Kms1 at amino acid 163 of SpTP, which is marked as an asterisk in Figures 2.9 and 2.10. Also, when searching Kms2 protein for Plk1 phosphorylation sites D/E-X-pS/pT-X, DLpTRE was found at amino acid 350 and EESSHE at amino acid 489 (circled P for putative phosphorylation site, Figure 2.9.B. and C., underlined in Figure 2.10).

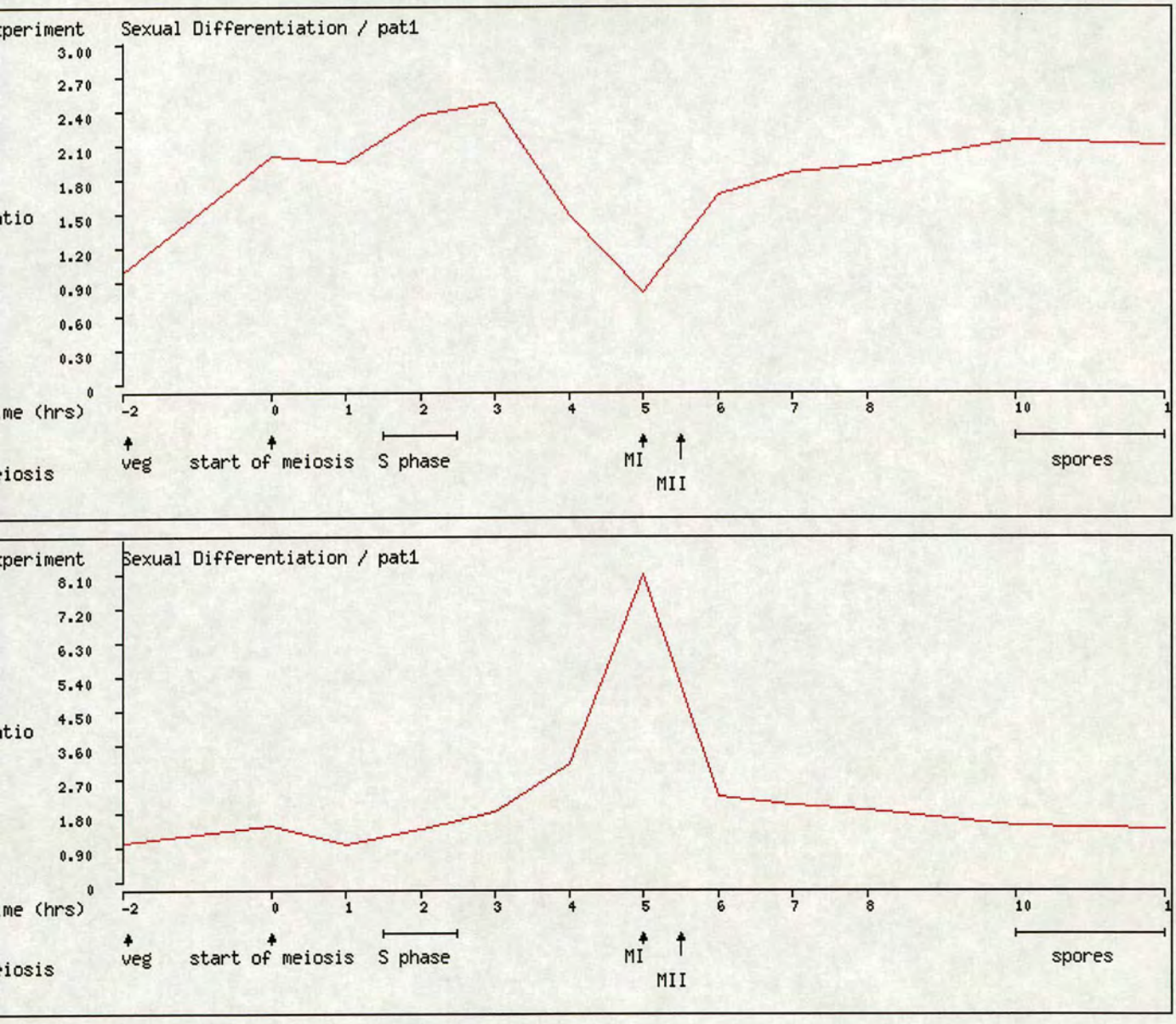


Figure 2.12. mRNA expression profiles of *kms1* and *kms2*.
Top) Meiosis profile of *kms1* mRNA expression in a *pat1* strain, showing a gradual rise until the beginning of meiosis, whereupon the mRNA levels drop to a low point at metaphase I and recovers after metaphase II.
Bottom) Meiois profile of *kms2* mRNA in a *pat1* strain, showing a sudden increase in mRNA levels at prophase I peaking at metaphase I and sharply decreasing after metaphase II.

2.9.c. Kms2

The Kms2 protein localises to the SPB throughout mitosis and meiosis. *kms2* and *kms1* are synthetically lethal, indicating that somehow both are required for vegetative growth (Shimanuki et al., 2002).

kms2 is not a meiosis-specific gene as it is expressed during mitosis. *kms2* expression varies with the meiotic cell cycle and peaks at meiosis metaphase I and then decreases at metaphase II (Figure 3.5. Bottom panel). *kms1* and *kms2* mRNA levels appear to be inversely correlated in this strain. The numbers discussed here represent relative levels, where 1.0 is the level of mRNA in the vegetative state prior to meiotic induction. *kms1* levels are higher (~2.0-2.5) during induction until the meiotic divisions at 5-6 hours, whereupon the mRNA levels decrease to 1.0. Then, *kms1* mRNA levels return to 1.5-2.0 after 6 hours during the meiotic divisions and sporulation (Figure 2.12).

kms2 mRNA expression shows a reverse pattern. *kms2* mRNA levels are similar to that of vegetative state until just prior to the meiotic divisions at 4-6 hours, whereupon they increase markedly to 7.0-8.1 and after which the levels decrease and remain low during sporulation (1.0) (Figure 2.12).

In a wild-type strain, *kms1* is expressed at high levels (2.5-3.5) during meiotic S phase and then dips at 5 hours, but returns to high levels (2.0) after 6 hours; *kms2* is similar to vegetative levels until about 4.5 hours, then rises during meiotic segregation to 2.0. *kms2* levels do drop at 10 hours, during sporulation. *plo1* expresssion follows a similar pattern to *kms2* with high levels at 5-6 hours.

In searching for homologues of Kms2, I found that Kms1 aligned with a value of $7e^{-10}$. Kms2 aligns well with Kms1 (www.t-coffee.org), even when the coiled-coil domains are removed ($1e^{-04}$) (Figure 2.10). When Kms2 is used, Kms1 scores higher probably because Kms2 is smaller at 457 amino acids, whereas when Kms1 is used for BLAST searches, it has sequences that are not aligned with any amino acids of Kms2 due to the length of Kms1 at 607 amino acids.

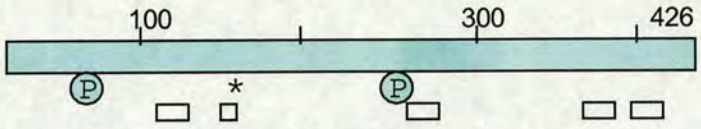
Kms2 protein has a transmembrane domain at amino acids 409-431, areas of low complexity at 357-369 and 371-386, as well as regions predicted to have a coiled-coil secondary structure at amino acids 170-211 and 360-387 (Figure 2.9.C). It has coiled-coil regions and an EF-hand at amino acids 7-31.

Kms2 has a putative binding site starting at amino acid 139, STP (* in Figure 2.9.C, also underlined in Figure 2.10). This aligns with STP of Kms1 at amino acid 163 (Figure 2.10). There are two SS sites at 207 and 384, and three ST sites at 67, 392 and 447. Two human Plk1 phosphorylation site exists in Kms2, one at threonine 79, ELpTGLD and the other at amino acid 206 DSpSEE, (P in Figure 2.9.C, also underlined in Figure 2.10).

2.9.d. Sum2

Sum2 refers to a suppressor of uncontrolled mitosis. This gene was identified from a screen aiming to find genes that restored the G2/M checkpoint in cells overproducing Cdc25. The N-terminal 372 amino acids of Sum2 were found to weakly rescue hydroxyurea-treated Cdc25-overexpressing cells, by preventing entry into mitosis (Forbes et al., 1998). However, the full-length protein of 426 amino acids was lethal in a Cdc25-overexpressing background when expressed from the *nmt1* promoter (Forbes et al., 1998). The localisation of Sum2 is not known. In another yeast two-hybrid screen with Plo1 as

A)



Sum2

B)

```
Sum2                ---MTEFIGSRISLISKSDIRYVGILQDINSQDSTLALKHVRWCGTEGRKQDPS 51
N.crassa            ---MSEFLGSRISLISRSDIRYVGTLHNINSEESTVSLENVRSFGTEGRKHNPD 51
Aspergillis_fum     -MDMNHLIGQRFNLISKSDIRYVGTLHEINPEASTIALENVVSFGTEGRRGNPA 53
Arabidopsis_tha     ---ADSYVGSLISLTSKSEIRYEGILYNINTDESSIGLQNVRSFGTEGRKKD-G 63
Arabidopsis_tha     ---ADSYVGSLISLTSKSEIRYEGILYNINTDESSIGLQNVRSFGTEGRKKD-G 63
Xen_laevis          MSGGTPYIGSKISLISKAEIRYEGILYTIDTENSTVALAKVRSFGTEDRPTD-R 53
Mus_musculus        MSGGTPYIGSKISLISKAEIRYEGILYTIDTENSTVALAKVRSFGTEDRPTD-R 53
Human               MSGGTPYIGSKISLISKAEIRYEGILYTIDTENSTVALAKVRSFGTEDRPTD-R 53
Conserved                 :*. :.* *:::*** * *  *:.: *::.* :*   ***.*  :

Sum2                QEIPPSDNVFDYIVFRGSDVKDLRIEEPATTPSAP-PVQPPNDPAIIGSNSGQ- 103
N.crassa            EEVPASDQVYEYIVFRGSDVKDLRIEEGPAPPKEN-KPPMPDDPAILGS----- 98
Aspergillis_fum     EEIPPSASVYEYIVFRGSDVKDISVAEEKKENAQPEPPRVPDDPAILGVSSTGP 107
Arabidopsis_tha     PQVPPSDKVYEYILFRGTDIKDLQVKASP--PVQPPASTINNDPAIIQSHYPSP 115
Arabidopsis_tha     PQVPPSDKVYEYILFRGTDIKDLQVKASP--PVQPPASTINNDPAIIQSHYPSP 115
Xen_laevis          P-IPPRDEVFEYIIFRGSDIKDLTVCEPP-----KPQCSLPQDPAIVQSSLGSS 101
Mouse               P-IPPRDEVFEYIIFRGSDIKDLTVCEPP-----KPQCSLPQDPAIVQSSLGSS 101
Human               P-IPPRDEVFEYIIFRGSDIKDLTVCEPP-----KPQCSLPQDPAIVQSSLGSS 101
Conserved             : *. . *::**:***:*:**: :              :****:
```

Figure 2.13. Alignment of proteins sharing homology with Sum2.
A) Sum2 has areas of low complexity throughout at amino acids 108-146, 149-159, 263-278, 357-369 and 384-401. Circled P's represent putative phosphorylation sites and the asterisk, a potential binding site.
B) Sum2 is aligned with a hypothetical 61 kD protein from *Neurospora crassa* (Q873J2), hypothetical 61 kD protein from *Aspergillus fumigatus* (Q8TGE7), a hypothetical 68 kD protein from *Arabidopsis thaliana* (Q9C604), a hypothetical 64 kD protein from *Arabidopsis thaliana* (Q9C658), a hypothetical 51 kD protein from *Xenopus laevis* (Q8AVJ2), an unknown protein predicted from mouse RIKEN cDNA of 50kD (Q8K2F8) and a human hypothetical 50 kDprotein (Q96SN5).

bait, Sum2 was found as a putative interactor (Reynolds and Ohkura, 2003). *sum2* mRNA is expressed during mitosis and throughout meiosis. There is a peak of *sum2* expression during meiotic S phase (www.sanger.ac.uk).

I searched for homologues of Sum2 and found that several hypothetical proteins from various species aligned well in the first 110 amino acids. Homologues from *Neurospora crassa* (Swiss-Prot ID: Q873J2), a suppressor of clathrin deficiency from budding yeast, Scd6 (P45978), and Rap55 from newt (Swiss-Prot ID: Q9YH12).

Alignments were performed for the proteins above $1e^{-19}$ and are shown in Figure 2.13.B. A region at the beginning of the protein showed homology (amino acids 1-103 in Sum2 and the C-terminus showed homology where RG-rich stretches exist (amino acids 384-402 in Sum2), which could be involved in binding RNA.

There is one STP site starting at amino acid 162 marked as an asterisk in Figure 3.7.A. There are no SS sites for binding in Sum2, but there are ST sites starting at amino acids 31, 257 and 263. Two Plk1 consensus phosphorylation sites exist in this sequence at serine 53 in DPpSQ and at serine 252 in EApSI (P in Figure 2.13.A).

2.10. The regions of Plo1 responsible for interaction with prey

Polo-like kinases have an amino-terminal catalytic domain and a highly conserved carboxy-terminal non-catalytic domain. Polo boxes exist in the non-catalytic domain in the C-terminal half of Plks. To see which part of Plo1 interacts with the prey proteins, various constructs were tested (Reynolds and Ohkura, 2003).

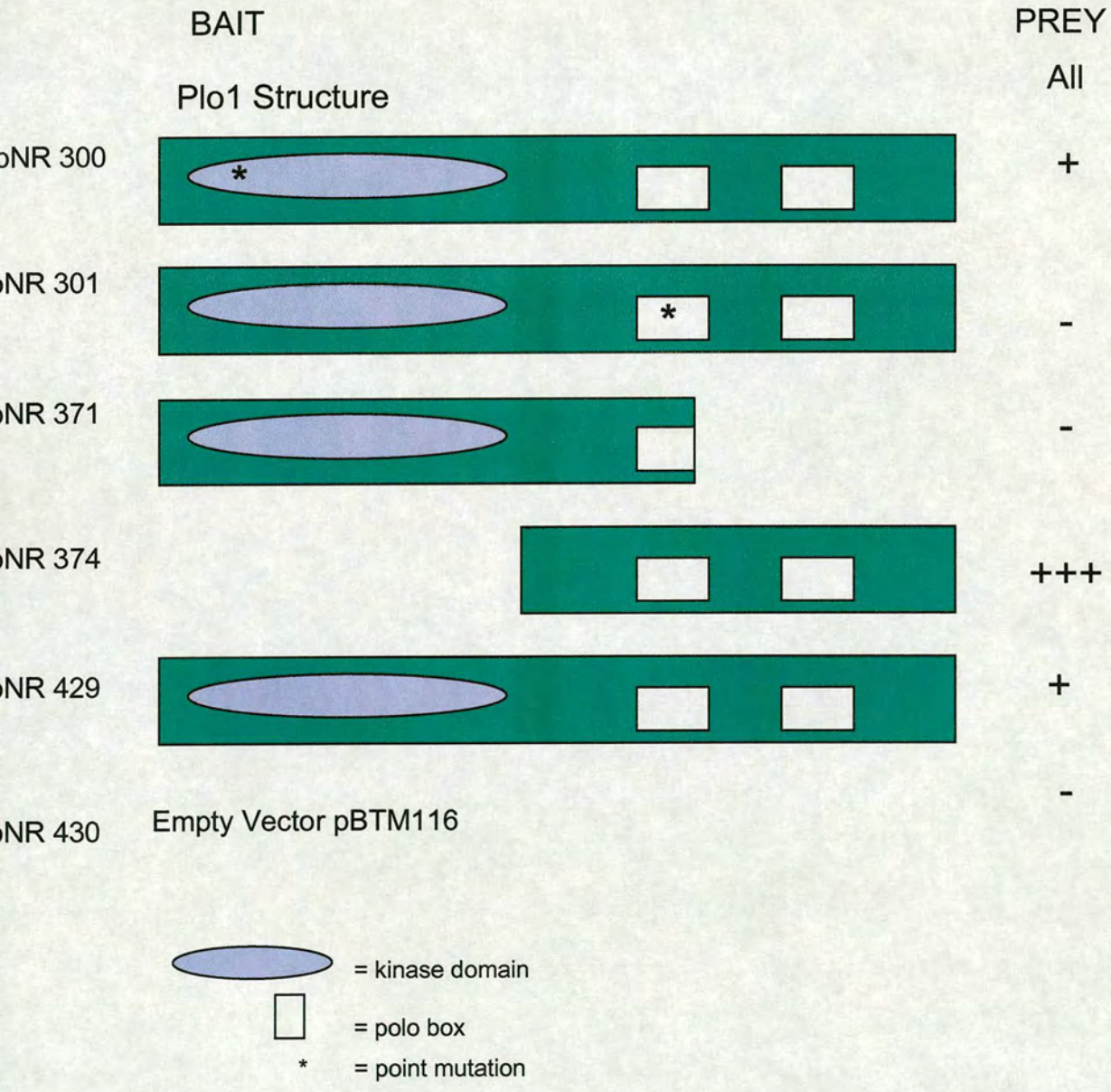


Figure 2.14. Polo boxes are responsible for interaction with all prey.
All interactors show the same affinity for various constructs of Plo1. The first point mutation in the kinase domain K69R does not affect binding of interacting proteins. The point mutation in the first polo box YQL508AAA and the deletion of the last two polo boxes abolish interaction with all nine interacting proteins. Deletion of the kinase domain results in stronger interaction.

All nine true interactors were tested with each construct and assayed for *lacZ* activity. The first construct pNR300, which had a point mutation in the kinase domain (K69R) that abolishes kinase activity, had no effect on binding of any of the interactors (Figure 2.14). The next two constructs, pNR301 that had a point mutation in the first polo box (YQL508AAA) or pNR371 in which the last two beta sheets of the first polo box and the second polo box were deleted (Δ 534-684), abolished binding with each interactor (Figure 2.14).

Deletion of the kinase domain in pNR374 (Δ 1-313) results in more intense *lacZ* activity (Figure 2.14). It is possible that the shorter protein is made more quickly and thus is more abundant. That could be why the kinase domain interacts more strongly, or that the kinase domain negatively regulates the polo box domain's interaction with other proteins. The polo box domain may be more exposed and then interact with more affinity. It also could be that the kinase domain inhibits the polo box domain from interacting. It is true that the polo box domain inhibits kinase activity and upon interaction, the inhibition is lifted resulting in increased kinase activity. The reverse could be true, where the kinase domain inhibits the polo box domain from interacting with other proteins. Overall, the results indicate that the polo boxes are responsible for interaction with all prey.

2.11. Key results from the screen for Plo1-interacting proteins

Nine proteins were identified as positive interactors of Plo1. Sum2 and SPAC26H5.05 had previously been found in a screen using a mitotic cDNA library (Reynolds and Ohkura, 2003). Thus, seven new proteins, Sid4, Kms1, Kms2, SPAC9.11, Htb1, Zym1 and

Ctt1, have been identified as potential Plo1-interacting proteins. The polo boxes are responsible for interaction with all nine interacting-proteins.

Chapter 3. Identification of Plo1-binding domains of the Spindle Pole Body Proteins and Disruption of the Sid4-Plo1 Interaction

3.1. Rationale for studying Kms1, Kms2 and Sid4

Since Plo1 localises to the SPB in a cell cycle dependent manner and Sid4, Kms1 and Kms2 also localise to the SPB, these proteins are of interest as interactors (Okazaki et al., 2002). The overall aim was to study the biological function of the interaction between the SPB proteins and Plo1. The approach used here has been to specifically disrupt the interaction with Plo1 by point mutation and to study the effects of perturbing the interaction.

3.2. Identifying the minimal domains of SPB proteins that bind Plo1

To disrupt the interaction, deletion constructs were made to determine the minimal binding domain within the SPB proteins for interaction with Plo1. These regions were then randomly mutagenised by PCR.

To construct deletion mutants, restriction sites were used to digest away a portion of the gene. The restriction sites used had to be sites that occurred within the cDNA and not within the vector. Deletions from the 5'-end or the 3'-end were made bearing in mind that the 5'-deletions would be designed so the deleted cDNA would be read in-frame with the Gal4 activation domain lying upstream of the cDNA. After deletion of DNA, the ends were ligated within the molecule. Restriction enzymes would leave compatible ends to be religated either without further modification or with Klenow treatment religation of one

blunt end and one compatible end, or two blunt ends could take place.

3.2.a. Creating *kms2* deletion constructs

The deletions of *kms2* cDNA are listed here. Digestion with SmaI and HpaI generated a 5'-deletion of 233 nucleotides (Figure 3.1) corresponding to an amino-terminal deletion of 76 amino acids. This left amino acids 77- 457, in-frame with the Gal4 activation domain embodied in construct A6 (Figure 3.1). The NotI/HpaI digest deleted 1141 nucleotides of the 3' end of *kms2* cDNA, resulting in amino acids 5-77 (Construct D10, Figure 3.1). The NotI/NarI digest would have resulted in a 3'-deletion of 422 nucleotides, but upon sequencing I found 564 nucleotides were deleted. The remaining region of *kms2* cDNA produced amino acids 5-273 amino acids (Construct G1, Figure 3.1). A further 5'-deletion of construct G1 resulted in construct M, which had amino acids 77-273, using SmaI and HpaI (Figure 3.1).

3.2.b. Assaying the *kms2* deletion constructs for binding with Plo1 bait

To test the interaction between Plo1 and the various Kms2 deletions, yeast strains containing *plo1* bait plasmid and *kms2* constructs were tested for interaction. The wild-type PB9 (amino acids 5-457 of Kms2) was used concurrently as a control. The interaction of wild-type Kms2 with Plo1 produced a weak blue colour upon testing for *lacZ* activity, indicating a weak interaction (Figure 3.1 one + sign indicates a weak interaction). Four transformed colonies were tested for each construct and compared with wild-type Kms2 for *lacZ* activity. The *lacZ* assays were performed a minimum of three times. Construct A6 (encoding amino acids 77-457) had varying results from weak to medium interactions over six different X-galactose assays. Construct D10, which encoded amino acids 5-77, was negative for interaction in each *lacZ* assay. Construct G1 that produces

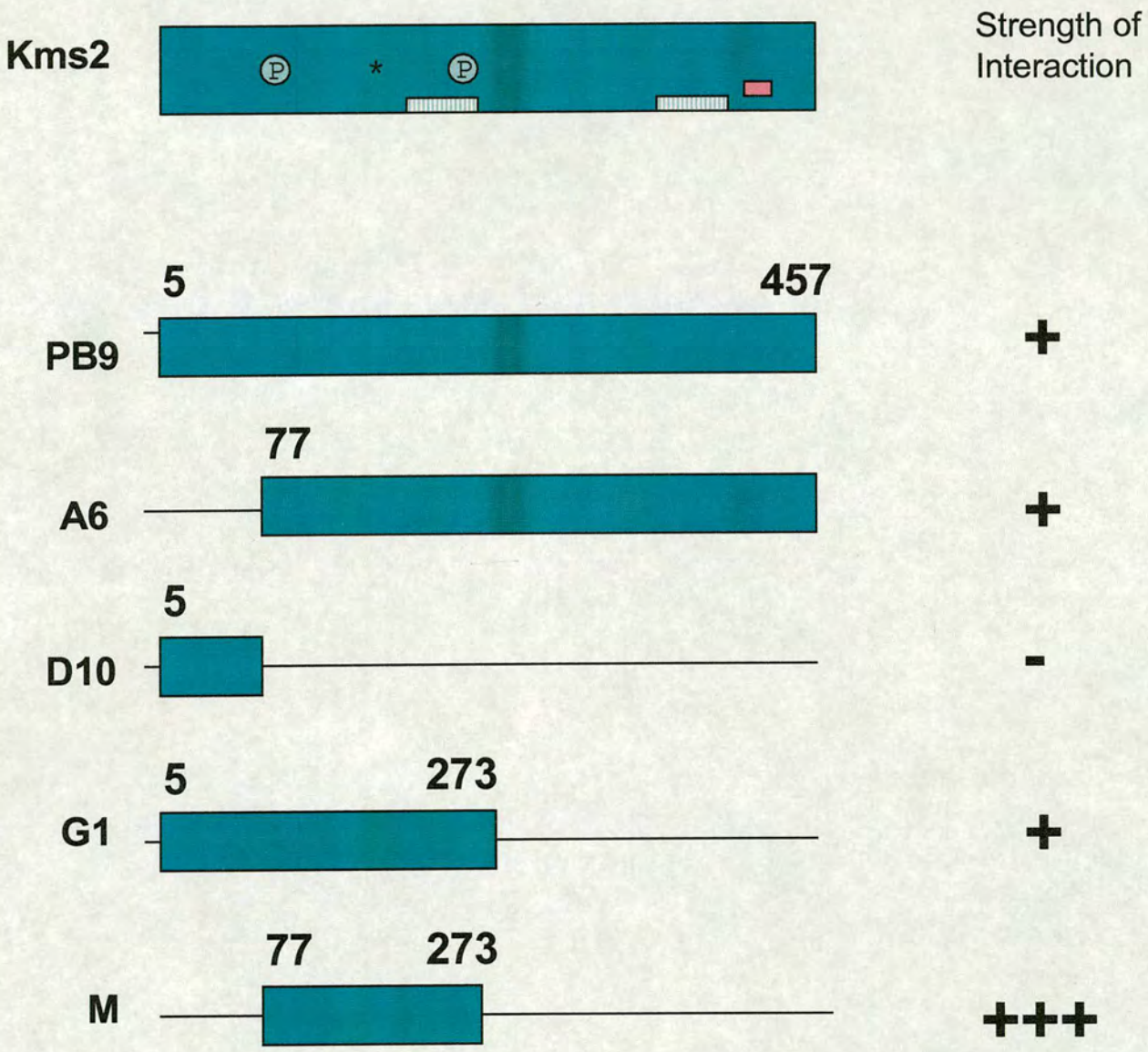


Figure 3.1. Deletion Constructs for Kms2.
The circled Ps represent putative phosphorylation sites and the asterisks, potential binding sites. The striped boxes represent coiled-coil domains, the pink boxes, putative transmembrane domains and the white box, an area of low complexity. The above constructs correspond to those in Figure 5.2.
A6=(Δ SmaI/HpaI, 77-457), D10= (Δ NotI/HpaI, 5-77), G1=(Δ NotI/NarI, 5-273), M=G1Δ SmaI/HpaI 77-273

amino acids 5-273 had a medium interaction with Plo1. Construct M, encoding amino acids 77-273, had a very strong interaction consistently over six assays for *lacZ* activity (Figure 3.1). Thus, the region of amino acids 77-273 of Kms2 was consistently sufficient for interaction with Plo1.

3.2.c. Creating *kms1* deletion constructs

There were difficulties making deletion constructs of *kms1* cDNA because of rearrangements in the plasmid. The SalI/XhoI deletion removed the first 623 nucleotides of *kms1* cDNA, which left amino acids 206-607 from the original 48-607 amino acids (Construct C1, Figure 3.2). Unfortunately, NotI cut the vector twice. An attempt, to eliminate the second NotI site by ligating the plasmid after cutting with NotI, was not successful. Rearrangements of plasmids can take place in yeast and this is a possible reason for *kms1* not being amenable to deletions.

3.2.d. Assaying the *kms1* deletion construct

To verify presence or absence of an interaction between Plo1 and the two Kms1 proteins, varying in length, yeast containing *plo1* bait plasmid and *kms1* constructs were tested for interaction. The wild-type PB6 (amino acids 48-607 of Kms1) was used concurrently as a control. The interaction of wild-type Kms1 with Plo1 produced a weak blue colour upon testing for *lacZ* activity, indicating a weak interaction (Figure 3.2). Four transformed colonies were tested for each construct and compared with wild-type Kms1 for *lacZ* activity. The *lacZ* assays were performed a minimum of three times. Construct C1 (encoding amino acids 206-607) had varying results from weak to medium interactions over six different X-galactose assays.

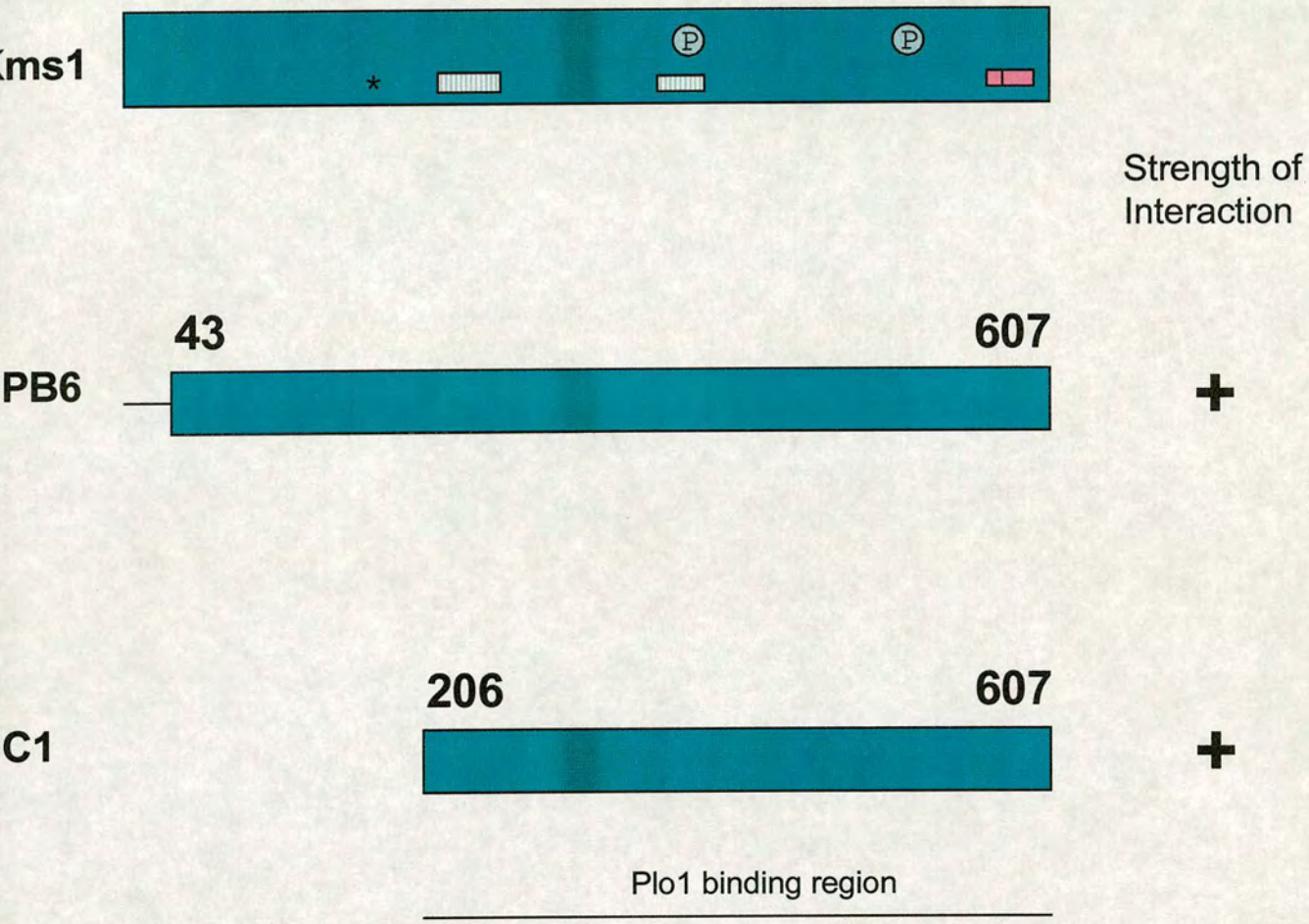


Figure 3.2. Kms1 deletion constructs. The above constructs correspond to those in Figure 5.2. C1=(Δ SalI/XhoI, 206-607). Above is a diagram of the putative domains of Kms1, including putative phosphorylation sites represented by circled P's, a potential binding site by an asterisk, areas of low complexity shown in white boxes, coiled-coil domains in the striped boxes and transmembrane domains are indicated by pink boxes.

3.2.e. Identifying the Plo1-minimal binding domain of Sid4

The deletion constructs of Sid4 are listed here. NotI and StuI were used to digest full-length *sid4* (PB7) to delete the last 268 nucleotides , leaving amino acids 1-572. Digestion with NotI and NdeI, 1525 nucleotides of the 3'-end were eliminated, resulting in a deletion construct encoding amino acids 1-153 (Construct I3, Figure 3.3). The EcoRI/NdeI deletion produces a protein with the first 153 amino acids removed, by making a 5'-deletion of the first 458 nucleotides (amino acids 153-660 in construct L10, Figure 3.3.). This construct was further deleted from the 3'-end using NotI and StuI, to delete the last 268 nucleotides. The remaining construct encoded amino acids 153-572 of Sid4 (N10, Figure 3.3).

3.2.f. Assaying the various deletion constructs for binding with Plo1

The constructs F6 (amino acids 1-572), I3 (amino acids 1-153), N10 (amino acids153-660) and L2 (amino acids153-572) were used to transform yeast and then were tested for interaction using the X-galactose overlay to determine if the yeast were *lacZ* positive. If they were *lacZ* positive, this indicated a positive result for interaction with Plo1 (Figure 3.3). The original *sid4* cDNA obtained from the screen (plasmid PB7) interacts strongly with Plo1, as does construct F6 encoding amino acids 12-572 of Sid4. Construct I3 encoding amino acids 12-153 cannot interact with Plo1 and deletion of this portion in plasmid L2 (amino acids 153-660) has greatly reduced *lacZ* activity in comparison to wild-type Sid4 (PB7). Amino acids 153-572 in construct N10 has similar activity to amino acids 153-660, though weaker when compared to PB7, but sufficient (Figure 3.3). Without the initial 153 amino acids, the interaction was much weaker. Amino acids 1-153 might be important for the intensity of the interaction or the stability of the protein, but this region on its own is not sufficient for interaction (Construct I3, Figure 3.3).

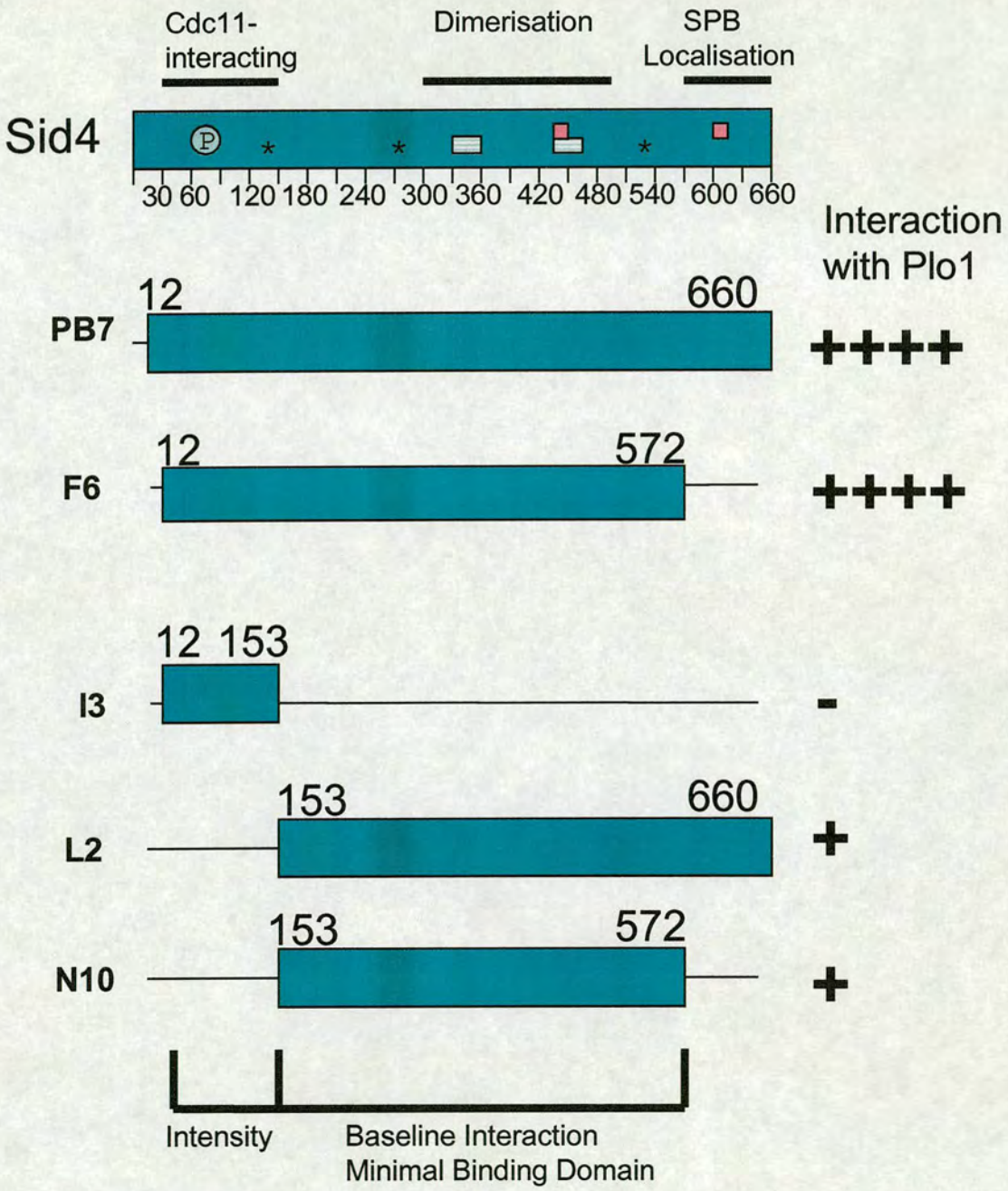


Figure 3.3. Deletion constructs that were made and tested for interaction with Plo1. The circled P represents a putative phosphorylation site and the asterisks, potential binding sites. The striped boxes represent coiled-coil domains, the pink boxes, putative transmembrane domains and the white box, an area of low complexity. Plasmid PB7 encodes amino acids 12-660 and interacts strongly with Plo1, as does F6, coding for amino acids 12-572. Construct I3 of amino acids 1-153 was not sufficient for interaction, but when deleted in constructs L2 and N10, reduced the intensity of the interaction with Plo1. F6 (ΔStuI/NotI, I3 (ΔNdeI/NotI), L2 (ΔEcoRI/NdeI), N10 Δ (L10StuI/NotI)

3.3. Isolation of point mutations in Kms1 and Kms2 that affect the interaction with Plo1

3.3.a. Random mutagenesis to synthesise point mutations

To make point mutations in Kms1 and Kms2 that would diminish binding with Plo1, the minimal binding domains of both proteins were subject to random mutagenesis by polymerase chain reaction (PCR). The minimal binding domain was subjected to random mutagenesis by PCR, assuming that Taq polymerase makes errors every 1/1,000 base pairs using conditions with 1mM dNTPs and 1.5mM $MgCl_2$.

3.3.b. Random mutagenesis of *kms2*

To disrupt the interaction of Plo1 with Kms2, the minimal binding domain was randomly mutagenised and tested directly in yeast using the gap-repair method (Figure 3.4). To select mutations that were altered for Plo1 binding, yeast strains were assayed for *lacZ* activity after random mutagenesis by PCR and gap-repair. For Kms2, amino acids 77-273, representing the minimal Plo1-binding domain was used for random mutagenesis. The oligos [KMS2 Forward (AA004) and KMS2 Reverse (AA005)] used encompassed the 591 nucleotides of the minimal binding domain plus flanking regions on either side. The PCR product was gel-purified and kept aside. The plasmid PB9 was sequentially digested with KasI and HpaI and gel-purified. Yeast containing bait plasmid was transformed with the linearised plasmid and the PCR product. Approximately 3150 colonies were examined. Single colonies were maintained and assayed for *lacZ* activity in comparison with wild-type control.

Out of the 3150 colonies tested, 25 colonies gave reduced *lacZ* activity compared to PB9.

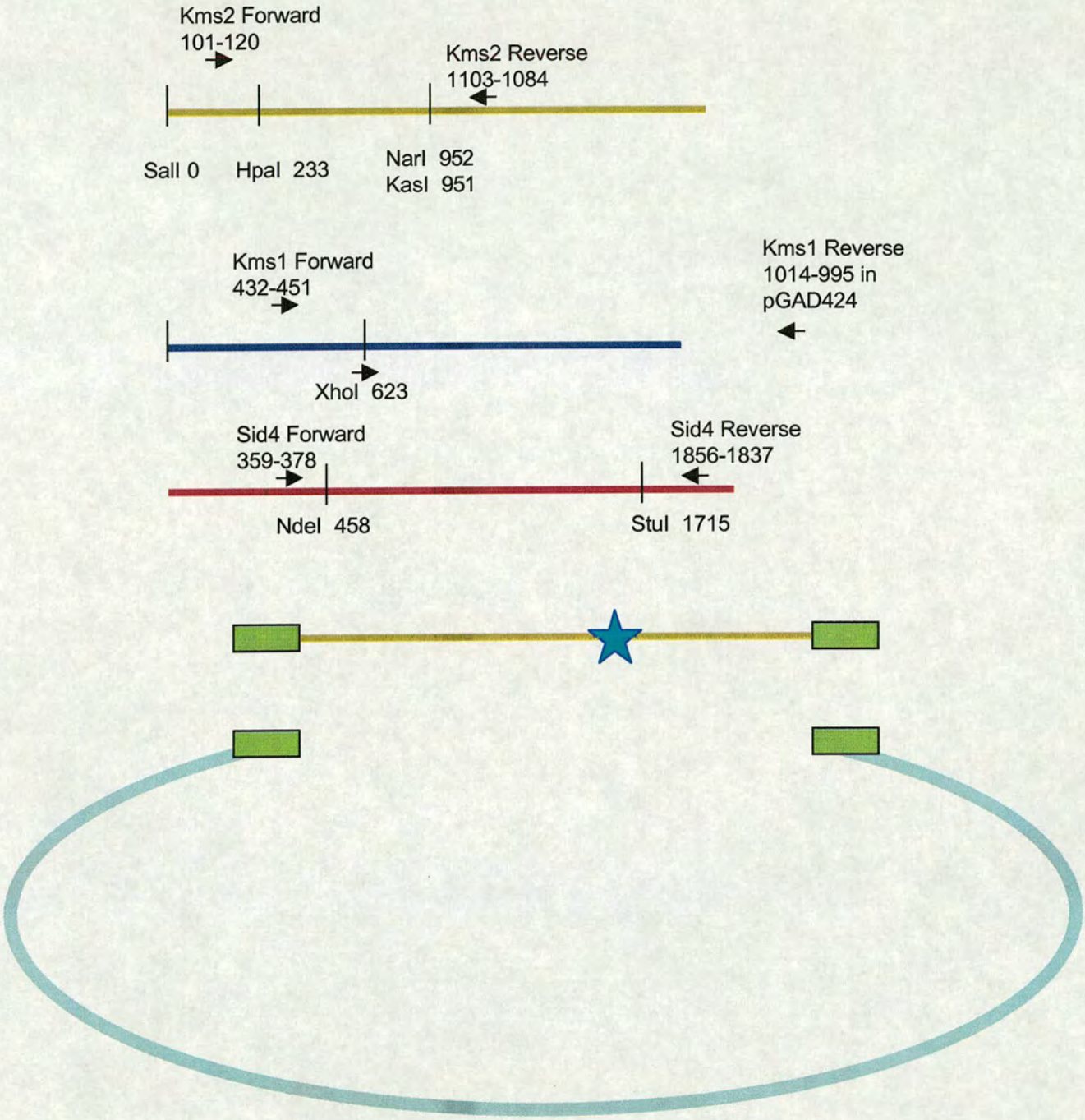


Figure 3.4. The Gap-Repair method. Using a standard method of PCR 1/1,000 mutations should occur. By making primers a PCR product with region homologous to approximately 100bp of the linearised plasmid's ends, the PCR product can recombine with the linear plasmid at the regions of homology to make a full-length plasmid again.

After single colony isolation, plasmid rescue and retransformation of yeast, two plasmids remained that gave little or no *lacZ* activity (M5-26w and M3-1). Sequencing revealed the mutations in these two plasmids. A 132 nucleotide-insertion was found in M5-26 (Figure 3.5). This results in 44 amino acids being inserted after amino acid 73. The 44 amino acids are a repeat of amino acids 71-114. M3-1 lacks amino acids 77-273 and is likely to be the cut plasmid recircularised (Figure 3.5). Thus, amino acids 77-273 were deleted, indicating this region is required for interaction with Plo1, besides being the minimal binding domain that is sufficient for interaction.

From all the transformations, 17 of 3150 colonies gave stronger *lacZ* activity than PB9 (the original kms2 cDNA from the cDNA library). They were isolated for single colonies, retested for *lacZ* activity and the yeast prey plasmids were recovered in bacteria after losing the bait plasmid first (as described in section 2.4). Then, the prey plasmid was purified from bacteria, yeast were retransformed with the plasmid DNA and bait plasmid containing Plo1 and retested for *lacZ* activity. Five out of 17 plasmids gave stronger *lacZ* activity than PB9. These five plasmids (A21, M5-19b, M5-8b, M5-5b and M5-3b) were sequenced and the mutations were identified (Figure 3.6). A21 and M5-19b each contain a point mutation which results in a premature termination codon that produces truncated protein. M5-8b also contains a point mutation that results in a premature termination codon, but it also has another mutation that mutates lysine to glutamic acid at amino acid 276. M5-3b has a one base pair deletion at nucleotide 7745, which results in a frameshift at amino acid 251. M5-5b had a one base pair insertion at nucleotide 7417, resulting in a frameshift at amino acid 339.

All of the mutations that exhibited a stronger interaction as assayed by *lacZ* activity resulted in truncation and removal of the C-terminal region (Figure 5.7). The truncated proteins may interact more strongly or they may be made more abundantly. If the former

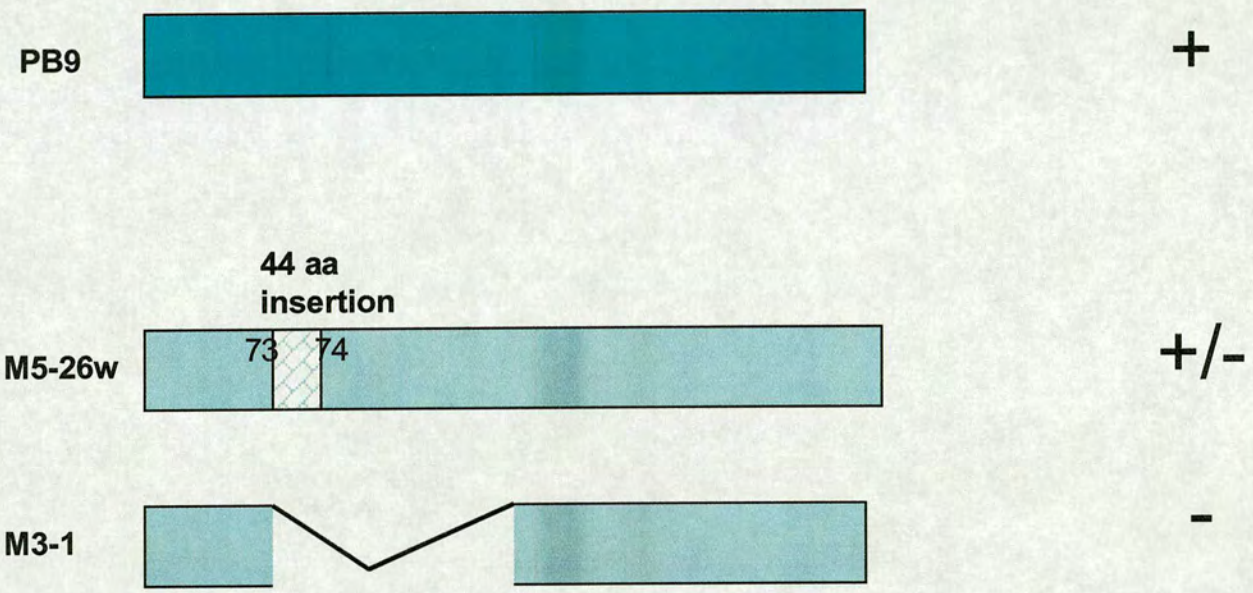


Figure 3.5. Kms2 point mutations were found to interact similarly to wild-type (M5-6b), and those that were quite pale blue, nearly white upon retransformation (M5-26w and M3-1).

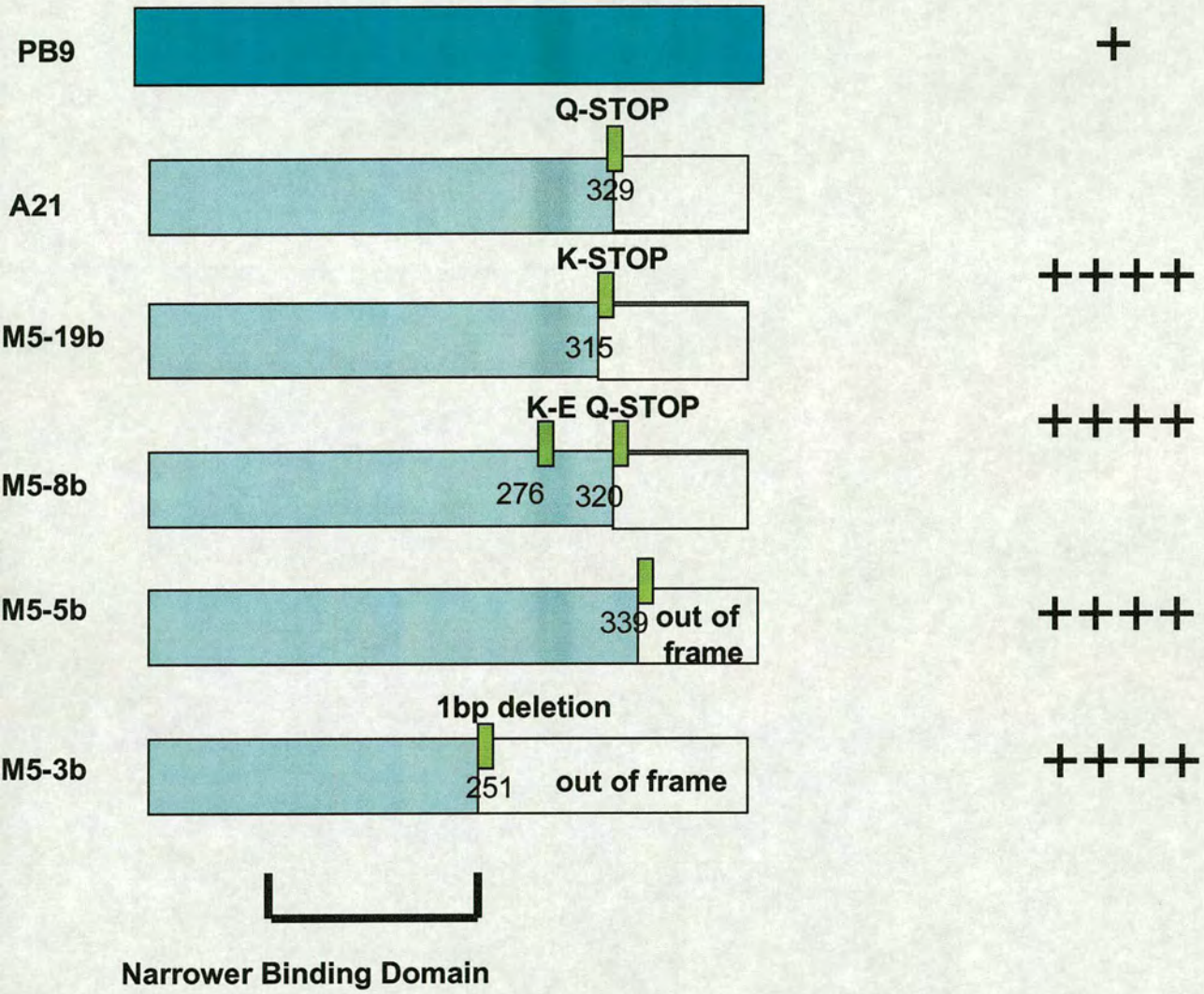


Figure 3.6. Kms2 point mutations were found that interacted more strongly with Plo1. Three constructs resulted in premature termination codons, which might eliminate a negative regulatory region for Plo1 binding. Finally, the point deletion at amino acid 251 that generates out-of-frame codons leads to a narrower binding domain.

explanation is correct, it is possible that a negative regulatory region for Plo1 binding exists in the C-terminal of Kms2. The latter reason could be true as the premature termination codons would produce smaller protein, and might be made more quickly and thus, more abundantly. This result narrows down the binding domain from amino acids 77-273 to 77-251.

3.3.c. Random mutagenesis of *kms1*

To disrupt the interaction of Kms1 with Plo1, random mutagenesis of the minimal binding domain 206-607 amino acids was performed by using oligos KMS1 Forward (AA006) and KMS1 Reverse (AA007), which flank the region encoding amino acids 206-607(Figure 3.4). Approximately 1800 colonies were tested for interaction with Plo1. There were many difficulties in rescuing the prey plasmids that resulted in darker blue colonies, which might be due to rearrangements in the plasmid. I was only able to obtain one plasmid from 40, and the isolated plasmid, which upon sequencing did not contain any mutations.

In summary, Kms2 has a narrower minimal binding domain of amino acids 77-251 than previously defined by deletion mutants. In addition amino acids 77-273 are required for interaction with Plo1. From this approach, disruption of the interaction between Kms2 and Plo1 by point mutation was not successful and since it was difficult to interpret the stronger intensity of the interaction at this point. Thus, Kms2 was not followed further with these methods. Random mutagenesis of Kms1 led to darker blue colonies, but the plasmids were not amenable to rescue. The loss of the plasmid might be toxic to the cell, or the plasmid may have recombined and integrated into the genome.

3.4. Study of the interaction of Plo1 with Sid4 in two-hybrid and in fission yeast

Sid4 functions in septation (Balasubramanian et al., 1998; Chang and Gould, 2000), as does Plo1 (Ohkura et al., 1995). Sid4 localises to the SPB and is responsible for all components of the SIN (septation initiation network) to localise to the SPB (Chang and Gould, 2000; Krapp et al., 2001). If Plo1 is not localised to the SPB, septation does not take place (Guertin et al., 2002), unless driven by another septation gene that acts downstream of Plo1 (the case referred to is Spg1 overexpression) (Mulvihill et al., 1999; Schmidt et al., 1997). The data points to the interaction between Plo1 and Sid4 being involved in septation. The aim of the following experiments was to determine the physiological function of the interaction. The approach was to disrupt the interaction by randomly mutagenising the minimal binding domain for Plo1 and determining the effects of the disrupted interaction in *Schizosaccharomyces pombe*.

3.4.a. Isolation of point mutants in Sid4 that alter interaction with Plo1

To determine what region of Sid4 is required for interaction with Plo1 and how a disrupted interaction might affect cellular functions, the minimal binding domain was mutated to perturb the interaction in budding yeast. Then the disrupted interaction was tested and compared to the wild-type Sid4-Plo1 interaction in fission yeast. The minimal binding domain was subjected to random mutagenesis by PCR, as it was shown that PCR with Taq polymerase, 1.5mM $MgCl_2$ and 1mM dNTPs results in approximately 1/1,000 mutations per base pair amplified (Reynolds and Ohkura, 2003). Then by having homology at the ends of the PCR product to a linearised plasmid, homologous recombination can take place in yeast (green boxes in Figure 3.4 are homologous regions). The gap-repair method refers to the technique in which investigators exploit the capacity for yeast to recombine linear segments of DNA. After transformation of yeast with linearised plasmid and PCR product, homologous recombination of both linear pieces of DNA allows each individual PCR product to be incorporated into prey vector.

To identify Sid4 mutants that had reduced interaction with Plo1, 5800 colonies were assayed for interaction with Plo1. The strains that exhibited weaker interactions, compared to the original Sid4, would be chosen for plasmid extraction (Figure 3.7). Once the plasmids were amplified by bacterial transformation and the plasmid DNA purified, these prey plasmids were used to retranform yeast containing Plo1 bait plasmid and compared with prey plasmid containing wild-type Sid4. The plasmids that exhibited consistently had weaker interaction were sequenced to find where mutations occurred.

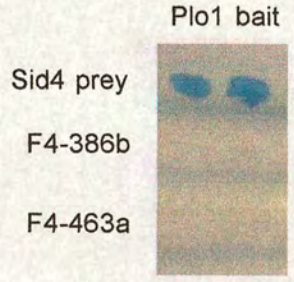


Figure 3.7. Sid4 interaction with Plo1. Sid4 prey interacts with Plo1 bait strongly, as measured by *lacZ* activity. The colonies exhibit a blue colour when subject to X-galactose assays. Two plasmids shown here, F4-386b and F4-463a, were mutated in *sid4*, disrupting interaction with Plo1. The colonies are white due to a lack of activity from *lacZ* reporter when exposed to X-galactose.

In total, ten mutants of Sid4 protein resulted in a disrupted interaction. Four of the mutants were due to premature termination codons. One mutant (F1-195) had a premature termination codon at amino acid 110, another mutant encoded by plasmid F2-qi terminated prematurely at amino acid 105 and plasmid F4-2497 contained a premature termination codon at amino acid 306 (Figure 3.6). Plasmid F4-386b had a premature termination codon at amino acid 225, as well as a silent mutation at amino acid 309 (Figure 3.6).

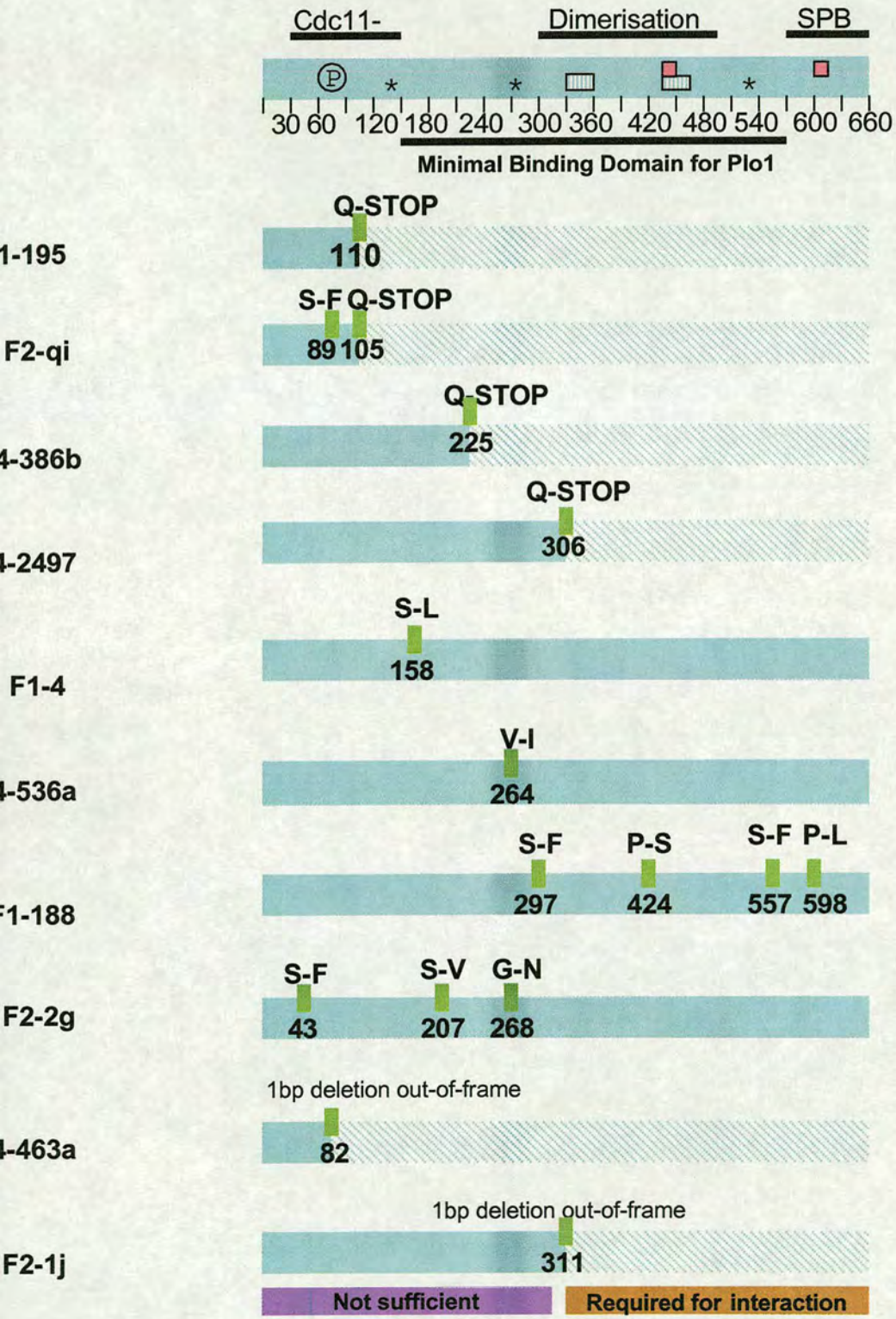


Figure 3.8. Sid4 point mutants generated by random mutagenesis that have reduced interaction with Plo1 when tested for *lacZ* activity. The circled P represents a putative phosphorylation site and the asterisks, potential binding sites. The striped boxes represent coiled-coil domains, the pink boxes, putative transmembrane domains and the white box, an area of low complexity. Four premature termination codons indicate that amino acids 1-306 of Sid4 are not sufficient for interaction with Plo1. Mutation leading to frameshift indicate that amino acids 311-570 are required for interaction. F1-188 and F2-2g have multiple mutations in the minimal binding domain, but single point mutations are specific, S158L and V264I, in F1-4 and F-536a respectively.

In addition to premature stop codons leading to truncated proteins, insertions or deletions can cause a frameshift. When one base pair is inserted or deleted, the cDNA is translated with the protein sequence altered after the point mutation, as the reading frame is shifted. Two out-of-frame mutations in Sid4 protein led to weaker interaction with Plo1. Plasmid F4-463a contained a 1bp deletion making the protein out-of-frame, thus leaving amino acids 1-82 as a truncated protein (Figure 3.8). Another mutant resulting from a 1bp deletion in plasmid F2-1j, produced an out-of-frame protein after amino acid 311. Amalgamating the information from deletion constructs and the information from the one base pair deletion after amino acid 311, indicating that amino acids 311-570 are required for interaction with Plo1.

Two plasmids had multiple mutations. Plasmid F1-188 had mutations at amino acids 297, 424, 557 and 598 and plasmid F2-2g had mutations at amino acids 43, 207 and 268 (Figure 3.8). In both constructs, more than one mutation occurred within the minimal binding domain. Thus, it is not clear which mutation is responsible for the weakened interaction with Plo1. Two other constructs had single mutations. Amino acid 158 was mutated from serine to leucine (S158L) in plasmid F1-4 and amino acid 264 was mutated from valine to isoleucine (V264I) in F4-536a (Figure 3.8). These are single mutations and thus the most minimal or least disruptive compared to either multiple mutations or out-of-frame mutations or premature termination codons. This type of point mutation disrupting the Sid4-Plo1 interaction is the aim of the approach was.

3.4.b. Attempts to assess the level of protein expression

The two point mutations of Sid4, S158L and V264I, have weakened interaction with Plo1, but I needed to ascertain that the mutations were the cause of reduced interaction. Although

it appears that Sid4 S158L and Sid4 V264I disrupt interaction with Plo1 (Figure 3.8), it is possible that less protein is synthesised, or what is made is less stable. The next aim was to determine the level of protein expression for the single mutations that disrupted the Sid4-Plo1 interaction.

I used the commercially available Gal4AD antibody to detect protein expressed from prey vectors. Protein was extracted from budding yeast containing mutant *sid4* or wild-type *sid4* in prey plasmid.

I tested wild-type *sid4* in plasmid PB7, the original cDNA from the meiotic library contained in prey vector pGAD424. At the same time, I used a protein that is detectable in the prey vector pACT2 (pACT2-*pol3*). PB7 showed no signal, but protein expressed from pACT2-*pol3* produced a strong chemiluminescent signal after 30 seconds of exposure to X-ray film (Figure 3.8). Exposure of up to an hour did not produce any signal for the Sid4 proteins. Consequently, it was not possible to detect Sid4 protein in this manner for assessing levels of wild-type Sid4, S158L Sid4 and V264I protein.

3.4.c. Assessing mutant Sid4 function

To assess whether Sid4 mutant proteins were able to perform functions other than Plo1-binding, I decided to investigate whether the mutants were capable of binding proteins that wild-type Sid4 is known to interact with.

Sid4 does not interact directly with components of the septation initiation network (SIN), but through Cdc11. Cdc11 has been reported to interact with Sid4 by yeast two-hybrid analysis as well as by immunoprecipitation (Krapp et al., 2001; Tomlin et al., 2002). I

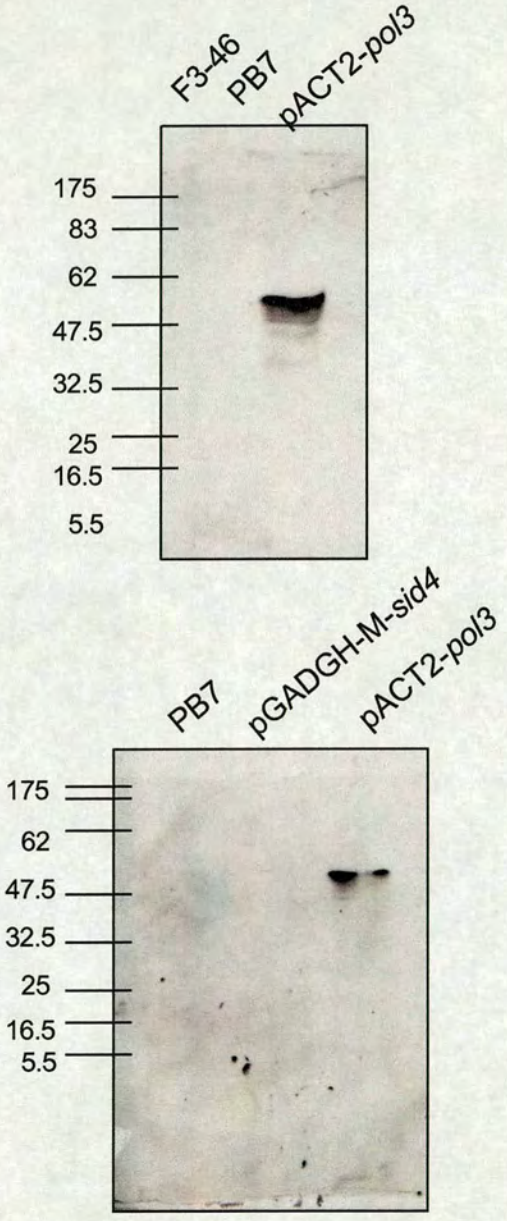


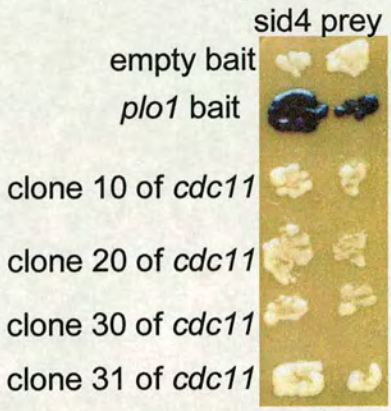
Figure 3.9. Attempt to assess Sid4 protein levels. Top panel. Attempt to measure the level of protein expression from wild-type and mutant Sid4. Bottom panel. pNR406 whose vector was pGADGH was not recognised by the Gal4AD antibody. Upon sequencing, 1kb of the promoter was missing. The full-length of *ADH1* promoter should have been present according to maps.

cloned *cdc11*$^+$ from genomic DNA of fission yeast. I had designed oligos that had restriction sites for cloning into bait vector. I amplified the *cdc11*$^+$ gene using Pfu/Taq with proofreading ability and then restriction-digested the PCR product. The PCR product was ligated into linearised bait vector. By sequencing four clones, I found that one had no errors in it. All four clones of *cdc11*$^+$ in bait vector (pGBT9) did not interact with wild-type *sid4*$^+$ in prey when tested for *lacZ* activity (Figure 3.10.A). The apparent discrepancy between my experiment and reported literature may be due to which gene is present in either bait or prey plasmid. *cdc11*$^+$ was in the prey vector (pGAD424) and *sid4*$^+$ in the bait vector (pGBT9) in reported studies (Tomlin et al., 2002). In this experiment, *sid4*$^+$ was in the prey and *cdc11*$^+$ in the bait vector, as all the mutations of *sid4*$^+$ were in prey vector pGAD424.

Sad1 has been reported to interact with Sid4 (Okazaki et al., 2001). I amplified wild-type *sad1*$^+$ from genomic DNA of fission yeast and cloned it into bait vector, as described above for *cdc11*$^+$. None of the five clones of *sad1*$^+$ interacted with *sid4*$^+$ prey.

It has been reported that Sid4 does interact with itself in yeast two-hybrid assays and in immunoprecipitation experiments (Chang and Gould, 2000; Tomlin et al., 2002). I cloned wild-type *sid4* cDNA into the bait vector, pGBT9. Transforming Y190 with pGBT9-*sid4* wild-type and PB7 (wild-type *sid4* in pGAD424 prey vector) or pGAD424-*sid4* mutant, I then tested for *lacZ* activity. The positive controls of wild-type *sid4*$^+$ in bait with wild-type *sid4* in prey showed that Sid4 interacts well with itself. I then tested the Sid4 proteins containing single point mutations S158L and V264I that have reduced interaction with Plo1. Sid4 single mutants that do not interact as well with Plo1 as wild-type, Sid4 S158L and Sid4 V264I interacted with wild-type Sid4 strongly, but not as well as compared to wild-type Sid4 (Figure 3.10.B). However, since it seemed that normal function of protein-protein interaction was not severely impaired, I decided to study these mutants further.

A)



B)

Figure 3.10. Attempt to determine the functionality of mutant Sid4 protein.
A) Interaction of Sid4 wild-type in prey vector with various clones of Cdc11. Clone 10 had no errors when the full-length gene was sequenced, but did not interact with Sid4 expressed from prey vector. B) Self-interaction of Sid4 wild-type and point mutant. The point mutants S158L and V264I are able to interact with Sid4 in bait. Appropriate controls are shown. Wild-type Sid4 expressed from prey interacts well with Sid4 expressed from bait, as well as with Plo1 expressed from bait. The mutant proteins can form heterodimers with wild-type Sid4.

3.4.d. Studying the disrupted Sid4-Plo1 interaction in fission yeast

To assess the possible biological function of the interaction between Sid4 and Plo1, I decided to investigate what effect the Sid4 proteins containing point mutations S158L and V264I would have in fission yeast. The mutants of Sid4 that no longer interacted with Plo1 could be compared with wild-type Sid4. I tested the function of Sid4 mutant proteins in a temperature-sensitive fission yeast strain of *sid4* (*sid4-SA1*) to reveal any defects caused by the lack of interaction between Sid4 and Plo1.

The mutant *sid4-SA1* contains a single point mutation at amino acid 629 changing a leucine to a proline (Chang and Gould, 2000). The *sid4-SA1* strain cannot localise any of the SIN proteins to the SPB at the non-permissive temperature and dies because of defective septation. It was shown previously that wild-type *sid4* could rescue *sid4-SA1* under the control of the *nmt1* promoter (Chang and Gould, 2000). I cloned wild-type *sid4* and two mutants of *sid4* into a plasmid with the *nmt1* promoter, with a C-terminal HA tag for the benefit of assessing levels of protein expression amongst wild-type Sid4 and mutant protein.

To determine whether the interaction was necessary for survival, the *sid4-SA1* was transformed with both mutant *sid4* genes *nmt1-sid4* S158L-HA or *nmt1-sid4* V264I-HA, or wild-type *nmt1-sid4*-HA. The empty vector was used to transform the *sid4-SA1* strain at the same time, as a negative control. The *nmt* promoter is a strong promoter to express proteins at high level and it is also repressible with increasing amount of thiamine. The *sid4-SA1* yeast strain was transformed with the plasmids and grown for 3-5 days at 25°C on EMM (minimal medium for fission yeast) with 2mM thiamine (2mM thiamine was used as per published results of Chang and Gould, 2000). Then, four colonies were patched and grown for one to two days at 25°C. Each patch representing individual colonies was

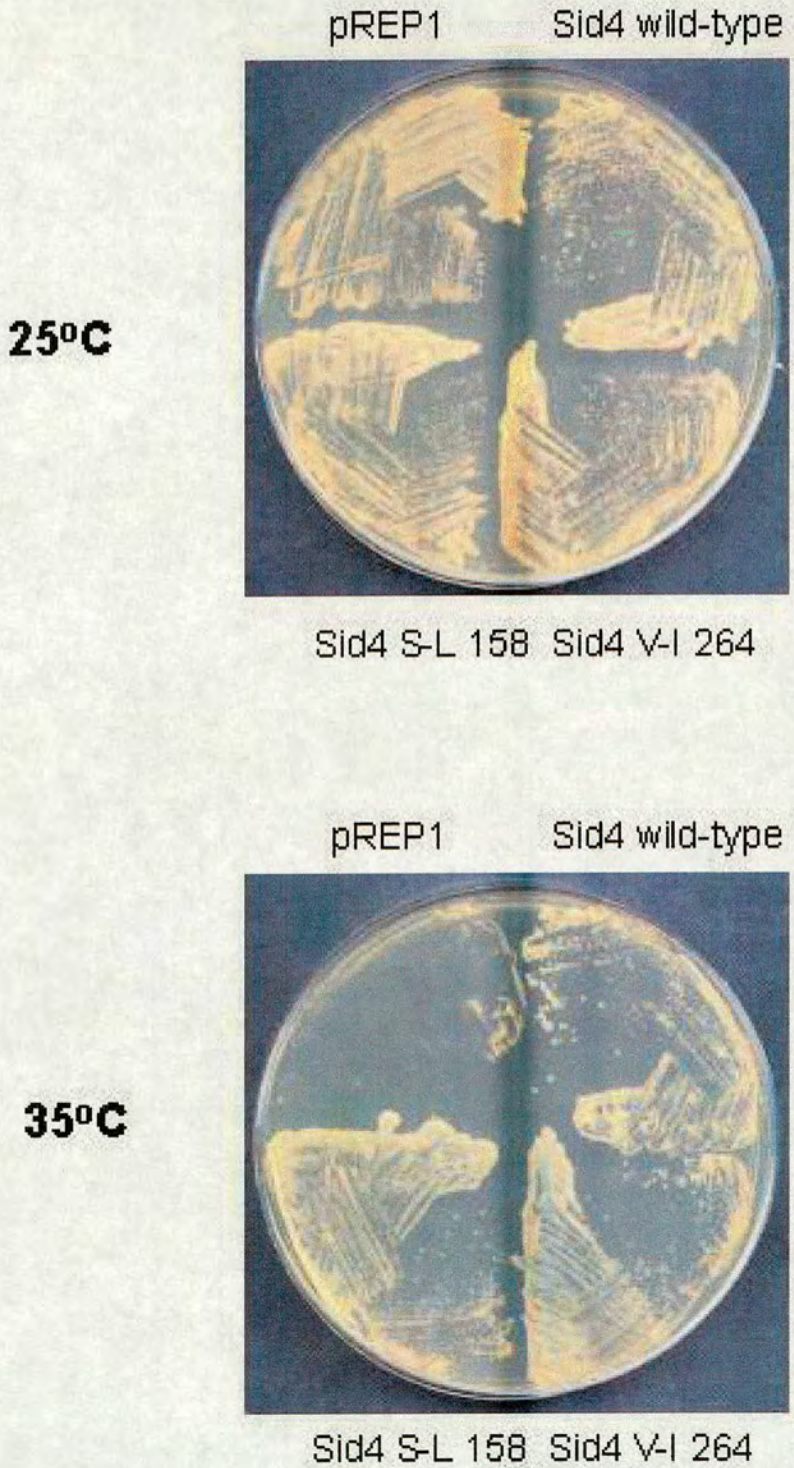


Figure 3.11. Rescue of *sid4-SA1*. The top panel shows constructs within pREP1 that were used to transform the *sid4* temperature-sensitive mutant strain (L629P *sid4-SA1*) at the permissive temperature, 25°C. Empty vector is at the top left, pREP1-*sid4* wild type at the top right. Both mutant *sid4* cDNAs in pREP1 are at the bottom of the plate.
The bottom panel shows the same constructs in *sid4-SA1* at the non-permissive temperature of 35°C. Both wild-type Sid4 and the point mutant S158L and V264I can rescue *sid4-SA1*.

streaked onto two different plates containing 2mM thiamine: one plate was incubated at 25°C, while the other was incubated at 35°C.

sid4-SA1 transformed with empty vector did not form colonies at the restrictive temperature of 35°C in contrast to growth at 25°C (Figure 3.11). Wild-type *sid4* cDNA (encoding amino acids12-660) allowed the temperature-sensitive strain to grow and form colonies at 35°C (Figure 3.11). Thus, Sid4 rescued the temperature-sensitive strain *sid4-SA1*, at the non-permissive temperature, consistent with the result by Chang and Gould (Chang and Gould, 2000). I tested the single point mutants of Sid4 that no longer interacted with Plo1 in yeast two-hybrid assays, Sid4 S158L and Sid4 V264I for growth at 35°C. Both *sid4-SA1* strains expressing either Sid4 mutant protein S158L or V264I formed colonies, indicating that they rescued the temperature-sensitivity of *sid4-SA1*.

Assuming that the results in two-hybrid assays reflect the situation in fission yeast, Sid4 would not interact with Plo1 and the above results would suggest that the Sid4-Plo1 interaction is not essential for septation. However, as Sid4 protein can form dimers (Chang and Gould, 2000), it is possible that Sid4 S158L or Sid4 V264I that no longer interact with Plo1, and the Sid4 L629P of the temperature-sensitive mutant, compensate for one another allowing rescue. Testing the disrupted interaction in a *sid4* null mutant would clarify whether the Sid4-Plo1 interaction is essential.

To assess the level of protein expressed, western blotting was performed.The Sid4 mutants and Sid4 wild-type were tagged with three HA repeats at the C- terminal of Sid4. Using an antibody to HA, western blotting was performed. The plasmids containing wild-type Sid4, Sid4 S158L and Sid4 V264I were used to transform *sid4-SA1*. The vector alone was also used to transform the *sid4-SA1*. Protein was extracted after growing cells overnight at 25°C. A cell pellet of 10^8 cells was resuspended in buffer containing with protease

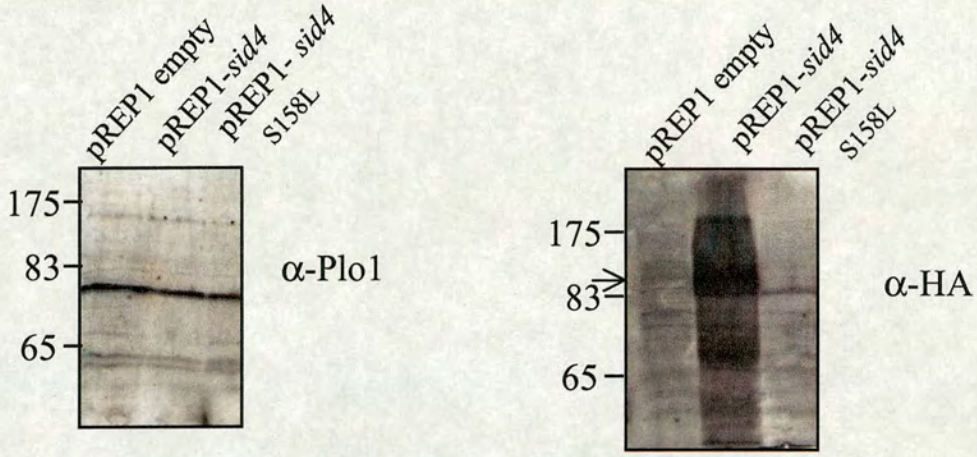


Figure 3.12. Determining the level of protein expression in *S. pombe* of wild-type Sid4 and mutated protein. Right-hand panel. The middle lane shows how wild-type Sid4 is expressed very strongly from the *nmt1* promoter. No band is observed in the first lane, as this represents the vector only. The point mutant of Sid4 S158L has approximately a 10x-reduction in protein level, shown in the first lane. Left-hand panel. Similar amounts of Plo1 were found in each strain.

inhibitor and glass beads, and shaken vigorously so that the cells would break open. Two aliquots of each protein sample were run on a denaturing gel, transferred to nitrocellulose and incubated with either anti-Plo1rabbit antibody or anti-HA mouse antibody. The two mutants Sid4 S158L expressed much lower levels of Sid4, although they had similar levels of Plo1 (Figure 3.12). Yet, there is sufficient protein to rescue the temperature-sensitive strain. Western blotting was repeated using protein extracted from an independent experiment, and the same result was observed for Sid4 S158L and Sid4 V264I.

Chapter 4. Discussion

4.1. The roles of Plo1 and interactors of Plks

Polo-like kinases regulate entry into mitosis, bipolar spindle formation, exit from mitosis and septation/cytokinesis (Nigg, 1998). To achieve such diverse functions, Plks are likely to interact with other proteins to mediate regulatory signals during the cell cycle. Some of the known Plk interactors mediate the functions of the polo-like kinases. For instance, Plk1 interacts with and phosphorylates NudC (Aumais et al., 2003; Zhou et al., 2003). Phosphorylation of NudC is required for cytokinesis in human cells (Aumais et al., 2003; Zhou et al., 2003). The same is true for Mklp2, which when Plk1 interacts and phosphorylates Mklp1, the phosphorylation enhances binding (Neef et al., 2003). The stronger interaction promotes decisive localisation of Plk1 to the midzone where Mklp2 localises, and this enables cytokinesis to occur properly (Neef et al., 2003). In *Drosophila*, the interaction between Polo and Pavarotti is necessary for cytokinesis to occur properly (Adams et al., 1998). The homologous proteins in human cells, Plk1 and CHO1/Mklp1 interact and play a role in cytokinesis; Lee, 1995 #105]. In budding yeast, interaction of Cdc5 with Scc1 and subsequent phosphorylation of Scc1 by Cdc5 allows for more efficient cleavage of Scc1 cohesin and initiation of sister chromatid segregation allowing for progression through mitosis. (Alexandru et al., 2001). Therefore, interacting proteins do mediate Plk function.

Meiosis-specific functions for Plks have been observed in mice (Pahlavan et al., 2000) and *Drosophila* (Riparbelli et al., 2000). Plks are responsible for spindle formation during meiosis in mammalian systems (Pahlavan et al., 2000; Riparbelli et al., 2000). Following depletion of Plk-1 by RNA-mediated interference in *Caenorhabditis elegans*, meiosis cannot be initiated, the nuclear envelope does not break down during oocyte maturation,

and after fertilisation, meiosis cannot be completed (Chase et al., 2000). The budding yeast Plk, Cdc5, is important for signalling for meiosis I, and for separation of homologous chromosomes to occur (Clyne et al., 2003; Cohen-Fix, 2003; Lee and Amon, 2003; Watanabe, 2003). Cdc5 interacts with proteins specifically expressed during meiosis, such as Rec8, to ensure appropriate separation of homologous chromosomes (Clyne et al., 2003; Lee and Amon, 2001; Lee and Amon, 2003). It is highly likely that conserved meiotic functions for the Plk, Plo1, exist in *Schizosaccharomyces pombe* also.

In fission yeast, three Plo1-interacting proteins have been identified and they do mediate specific Plk functions. Plo1 interacts with Cut12 to promote entry into mitosis (MacIver et al., 2003; Mulvihill et al., 1999). Plo1 also interacts with the anaphase-promoting complex to encourage progression through the cell cycle. Plo1 interacts an APC component, Cut23, to activate the APC, which leads to exit from mitosis (May et al., 2002; Reynolds and Ohkura, 2003). In addition, Plo1 interacts with Dmf1/Mid1, which is required for appropriate placement of the CAR and for the future site of cell division. Plo1 drives septation/cytokinesis, but it is not known how (Bähler et al., 1998; Mulvihill et al., 1999; Ohkura et al., 1995; Reynolds and Ohkura, 2003; Tanaka et al., 2001). Plo1 is responsible for spindle formation also (Ohkura et al., 1995; Reynolds and Ohkura, 2003). The molecules that Plo1 regulates have not been characterised. Therefore, it is crucial to identify Plo1-interacting proteins.

Currently, the role of Plo1 in meiosis in fission yeast is not known. In *Schizosaccharomyces pombe*, Plo1 has been screened for interactors using the two-hybrid method with a mitotic cDNA library as prey (Reynolds and Ohkura, 2003). In this study, the yeast two-hybrid method was used in hope of identifying Plo1-interacting proteins using a meiotic cDNA library.

4.2. The use of the two-hybrid method for screening Plo1-interacting proteins addressing the initial aims

I obtained nine proteins from the yeast two-hybrid screen using Plo1 as bait. The initial aims of this screen were 1) to find interactors that mediate Plo1 regulation of the diverse functions in the cell cycle, and 2) to find meiosis-specific interactors. The first aim was achieved, as seven new proteins were found to interact with Plo1 (Sid4, Kms1, Kms2, SPAC9.11, Htb1, Zym1, Ctt1). Two interacting proteins had been found before by yeast two-hybrid screening. These were SPAC26H5.05 and Sum2 (Reynolds and Ohkura, 2003). By using the microarray data available through the Sanger Centre's website (Chen et al., 2003; Mata and Bahler, 2003), it was observed that all nine proteins were expressed in mitosis, as well as in meiosis. Therefore, the second aim was not fulfilled as none of the genes were meiosis-specific, although *kms1* and *kms2* are important for meiotic functions.

The advantages of the two-hybrid technique include 1) the ability to screen a large number of proteins for interaction with one protein of interest, 2) the ability to detect scarce proteins, or transient interactions, and 3) the method is fast, simple and inexpensive. For Plo1, a kinase has very transient interactions, which the yeast two-hybrid method can detect. Another benefit is that the investigator does not need to have any previous knowledge of the proteins assayed, so one can find novel interactions. In addition, the method has been used successfully for Plo1 to identify biologically relevant interactors, such as Cut23 (May et al., 2002) and Mid1/Dmf1 (Bähler et al., 1998; Reynolds and Ohkura, 2003). Budding yeast Cdc5 and Dbf4 interact by two-hybrid assay and were found to genetically interact in earlier studies (Bartholomew et al., 2001). The yeast two-hybrid method has also been used successfully in this laboratory (Reynolds and Ohkura, 2003).

There are disadvantages to the two-hybrid method, in that false positives are often identified. False positives can occur within the yeast-two hybrid system because the prey may activate transcription without interaction with bait. Retransforming prey with bait and empty vector can eliminate false positives. Upon retesting for *lacZ* reporter activation, the result should be negative if the prey protein is a true interactor. If the result is positive, the prey protein is activating the reporter on its own. Those prey that are positive for *lacZ* with empty bait vector represent false positives. Out of 49 positives, I only found one false positive, *gpd3*. In addition, non-specific interactions are often identified. These interactions do take place in the yeast two-hybrid system, but may not in another type of experiment. The reasons are not entirely known for why non-specific interactions are identified frequently (Uetz et al., 2000; Uetz and Hughes, 2000), but a protein might be produced at high levels, and then interact non-specifically with bait protein. Interactions may also occur within the yeast two-hybrid system and in other types of experiments, such as immunoprecipitation, but may not occur in a biological context.

The other disadvantage of the yeast two-hybrid system, is that there may also be a lack of proteins identified that have putative transmembrane domains, as they may not be able to enter the nucleus to activate transcription (Uetz et al., 2000; Uetz and Hughes, 2000). Of the nine proteins identified in this study, three had transmembrane domains. Kms1, Kms2 and SPAC26H5.05 have small transmembrane domains of 20-40 amino acids. The nature of the transmembrane domains for these proteins may not have prevented nuclear import.

4.3. Relevance of interaction of Plo1 with proteins of unrelated function

In this study, full-length Plo1 was used as bait. The kinase domain does not appear to be required for interaction with other proteins for initial interaction (this study; (May et al., 2002; Reynolds and Ohkura, 2003). It is the C-terminal half, containing the polo boxes,

which is essential for interaction and sufficient for interaction with various proteins (this study; Jang, 2002 #54; May, 2002 #39; Reynolds, 2003 #89]. The polo boxes, when mutated at a single amino acid or deleted in part, could no longer interact with interacting-proteins (Reynolds and Ohkura, 2003). The mutations abolished interaction with all of the interactors identified in this study also. Recent data indicates that the C-terminal half of Plo1, containing the polo boxes, interacts with amino acid sequences containing phosphorylated residues of serine or threonine (Elia et al., 2003a; Elia et al., 2003b). In one instance, Plk1 recognises and binds human Mklp1 more tightly when phosphorylated (Neef et al., 2003). As full-length Plo1 was used as bait successfully before (Reynolds and Ohkura, 2003), it was more prudent to use full-length Plo1. Evidence now indicates that the polo cap and linker between the catalytic and non-catalytic domains are required in addition to the polo boxes for interactions (Elia et al., 2003a). The constructs used to test interaction were not mutated in the linker region, but one construct that had the kinase domain deleted, but included the linker, was sufficient to interact with all nine interactors.

The C-terminal half of Plo1, when expressed from bait vector, in conjunction with interacting prey proteins had higher *lacZ* activity than that of full-length Plo1 (Figure 2.14). From this result, if it is because the interaction is stronger and not due to altered protein levels, the PBD may be more exposed to interact with a higher affinity. The kinase domain may negatively regulate the PBD's capacity for interaction. On the other side of the coin, the polo box domain does negatively regulate kinase activity (Elia et al., 2003b). Upon interaction, inhibition of the kinase domain by PBD is lifted and kinase activity increases (Elia et al., 2003b).

Considering that the polo box domain in human Plk1 does recognise a consensus sequence, but cannot bind it with great affinity unless it is phosphorylated, could indicate that there is variability in what structure the PBD recognises. Often, very abundant

proteins can be present and would come into contact with the bait protein for its high concentration rather than a high affinity for such a protein. With respect to obtaining non-specific interactors, this does occur frequently in yeast two-hybrid screens.

Putative interacting-proteins that are very abundant include the histone H2B1, Ctt1 catalase and the metallothionein, Zym1. These genes are expressed at high levels. H2B1 does have cell cycle associated functions and could be relevant. Catalase and metallothionein, *ctt1* and *zym1* respectively, are housekeeping genes. They maintain the cellular environment free of toxic metabolites, such as metal ions and hydrogen peroxide. These functions have never been associated with Plo1 previously and they are probably not biologically relevant interactions.

SPAC26H5.05 and SPAC9.11 are two proteins that have not been characterised at all and were found as interactors. My sequence analysis indicates that SPAC9.11 has a coiled-coil region and that SPAC26H5.05 has ankyrin repeats, a transmembrane domain and an immunoglobulin-like fold. Two transcripts of SPAC 9.11 were found to interact with Plo1 (Table 2.3 and Figure 2.6); however, there were five stop codons upstream of the start codon in one of the transcripts. It is unlikely for such a cDNA to be able to produce a fusion protein with the Gal4AD, but it is not clear why the cDNA would result in a strong blue colour during X-galactose assays, indicating that the *lacZ* reporter was activated. To determine whether the fusion protein was being made, another tag could be cloned and checked for by western detection. With respect to SPAC26H5.05, Plk1 has been reported to interact with a Golgi protein containing ankyrin repeats, GRASP65 (Barr et al., 1997; Lin et al., 2000; Sutterlin et al., 2001). SPAC26H5.05 as was found as a Plo1-interacting in a yeast two-hybrid screen using a mitotic cDNA library as prey (Reynolds and Ohkura, 2003). Ankyrin repeats interact with a wide diversity of proteins, and are found in numerous disparate proteins and the SPAC26H5.05-Plo1 interaction may be non-specific.

Transmembrane domains and immunoglobulin-like folds are common in proteins with unrelated functions. Immunoglobulin-like folds are associated with protein-protein interactions for cell surface receptors and DNA-protein interactions for transcription factors (Pfam database accession number: PF01833). The coiled-coil domain of SPAC9.11 is the only distinctive feature of this protein. Therefore, the function of SPAC26H5.05 and SPAC9.11 could not be extrapolated by sequence data.

For all the proteins that have no related or no known function, further experiments would need to be performed to assess their relevance. To find if the interaction was occurring within fission yeast, immunoprecipitation experiments could be performed from protein extracts of wild-type fission yeast. In addition, genetic experiments could indicate whether the two proteins of an interaction might contribute to one physiological pathway. For this, overproduction of Ctt1, Zym1, SPAC9.11 or SPAC26H5.05 could be monitored for rescue of conditional *plo1* mutants. The reverse experiment could also be performed. If mutating the genes to disrupt interaction with Plo1 gave an altered phenotype, one could then examine if overproduction of Plo1 could diminish the observed phenotype.

4.4. Analysis of the identified cell cycle associated interactors of Plo1

To determine which of the nine interactors mediate Plo1 function, I collected information about each protein. Five of the interactors could have roles in the cell cycle, Htb1, Sum2, Sid4, Kms1 and Kms2.

Htb1 is a fission yeast histone H2B-α protein that interacts with DNA to package it (www.sanger.ac.uk). Plo1 does associate with chromosomal centromeres during the cell cycle (Nigg, 1998; Nigg et al., 1996). A possible future experiment would be to check if Plo1 and Htb1 colocalise. This would shed light on whether there is a cell cycle dependent

localisation of Plo1 to chromosomal proteins. In human cells, Plk1 does interact with BRCA2 and Chk2 (Lin et al., 2003; Tsvetkov et al., 2003).

The first 122 amino acids of Sum2 protein when expressed from the *nmt1* promoter weakly rescues the lethal phenotype of Cdc25 overproduction (Forbes et al., 1998). It is not known how Sum2 affects mitosis, particularly because full-length Sum2 protein is lethal in these experiments. Sum2 does suppress uncontrolled mitosis induced by Cdc25 overproduction and Sum2 may achieve this by inhibiting Cdc25. As Plks interact with Cdc25 (Qian et al., 1998; Qian et al., 1999), cyclin B1 (Yuan et al., 2002), Myt1 (Nakajima et al., 2003) and Wee1 (Bartholomew et al., 2001) in other species, it is possible that Plo1 may interact with Sum2 to control entry into mitosis. Plo1 does interact with Cut12 to rescue *cdc25-22cut12-s1* cells, by making the SPB more active and causing entry into mitosis (Bridge et al., 1998; MacIver et al., 2003). In addition, Sum2 was identified as a Plo1-interacting protein in another yeast two-hybrid screen using a mitotic cDNA library (Reynolds and Ohkura, 2003). It is not clear why Sum2 would be found to interact, but it is possible that there is an interaction that is real, but may not be biologically relevant. The potential binding site at amino acid 163 could mimic the peptide recognised by Plk1, which may also be recognised by Plo1 (Figure 2.13.A and section 2.9.d). To determine whether there is an interaction, an immunoprecipitation would be necessary, using fission yeast protein extract. Another type of experiment that could be helpful in determining whether the interaction is biologically relevant would be a synthetic lethal experiment. During the course of this work, the known *plo1* mutants were essential (Reynolds, 2001; Reynolds and Ohkura, 2003). Now, there are two mutants of *plo1* that can survive, *plo1-ts4* and *plo1-ts19* (MacIver et al., 2003). To delete *sum2* in combination with these temperature-sensitive mutants and observe whether they survive at a non-permissive temperature, would indicate if they are involved in a similar pathway.

Sid4, Kms1 and Kms2 are spindle pole body proteins. Since Plo1 localises to the SPB, these proteins were studied further. They are discussed in the following section 4.5.

4.5. Proteins that may be involved in Plo1 regulation of the cell cycle, due to their association with the SPB and other cellular processes

Three proteins, Sid4, Kms1 and Kms2, were found as novel interactors of Plo1 relate to functions associated with the cell cycle and localise to the SPB. Sid4 is required for septation (Chang and Gould, 2000). Kms1 is required for the integrity of the SPB during meiosis (Shimanuki et al., 1997). Kms1, Kms2 and Sid4 colocalise with Sad1, a known SPB component, and they localise to the SPB throughout the cell cycle (Chang and Gould, 2000; Shimanuki et al., 1997; Shimanuki et al., 2002). As Plo1 localises to the SPB for part of the cell cycle (Bähler et al., 1998), the SPB proteins were studied further.

4.6. Plo1 interactions with Kms1 and Kms2

Kms1 and Kms2 are homologous and they share 28% similarity at the amino acid level (Shimanuki et al., 2002). *kms1* is synthetically lethal with *kms2*, indicating a functional interaction between them (Shimanuki et al., 2002). The proteins Kms1 and Kms2 have also been found to interact by yeast two-hybrid assay (Miki et al., 2004). In this study, the yeast two-hybrid screen revealed two transcripts of *kms2* cDNA and one transcript of *kms1* cDNA to encode proteins that interacted with Plo1 bait.

During meiosis, microarray data indicate levels of mRNA expressed from genes (www.sanger.ac.uk; Mata, et al., 2003). A temperature sensitive fission yeast strain, *pat1*, allows for synchrony during meiotic induction. *kms1* mRNA expression has a pattern during meiosis that is expressed high prior to and after the meiotic divisions, but dips

during the meiotic divisions. *kms2* mRNA expression appears to be inversely correlated with kms1, in that there is a peak of expression during the meiotic division and levels similar to that of a mitotic state before and after the meiotic divisions (Figure 2.12). *plo1* expression levels during meiosis mimic the expression pattern exhibited by *kms2*. *plo1* levels are similar to vegetative state until 5-6 hours where mRNA levels increase to 4.0-5.4 during meiotic division and then return to 1.0-1.3 afterwards.

In the yeast two-hybrid screen using *plo1* as bait, *kms2* was found in fourteen strains, whereas *kms1* was only found in one strain. The meiotic cDNA library was made from mRNA extracted from a diploid wild-type fission yeast strain (see section 2.3). The mRNA was extracted at timepoints 2, 4, 6, 8 and10 hours after meiotic induction. In wild-type strain, meiotic landmark events do occur at a similar timepoints, but the synchrony is not as marked as in *pat1* strains. In a wild-type strain, *kms1* still has the reverse pattern of *kms2*, but not as markedly as in a *pat1* strain. The *plo1* expression pattern is similar to that of *kms2*, both of which have peak mRNA expression at 6x and 8x respectively, higher than that of mitotic mRNA expression at 5-6 hours after meiotic induction (www.sanger.ac.uk; Mata, et al., 2003). *plo1* mRNA might be enriched in the meiotic cDNA library from the 4 hour and 6 hour timepoints and *kms2* might be as well. Unfortunately, the microarray data published does not indicate the absolute quantity of mRNA and northern blotting of *in situ* hybridisation studies have not been performed for *kms1*, *kms2* or *plo1* during meiosis.

In addition, mRNA levels are not known for synchronised fission yeast going through the mitotic cell cycle, although Plo1 protein levels are constant (Mulvihill et al., 1999). It does appear that *kms2* and *plo1* mRNA expression patterns are similar in meiosis, both in a wild-type strain and in a *pat1* strain. It is possible that higher mRNA levels and subsequently higher protein concentration contributes to the presence of an interaction

between Plo1 and Kms2 during the meiotic divisions. The yeast two-hybrid data indicate an interaction occurring within budding yeast. To understand whether the interaction does occur and its biological significance, immunoprecipitation in fission yeast would need to be performed. It would be extremely useful to synchronise yeast undergoing meiosis and have protein extracts from different timepoints, particularly at 4-6 hours to assay the amount of Plo1 and Kms2 produced and also, for their interaction. The same type of experiments could be done for Plo1 and Kms1 protein, but a focus on production during meiotic S phase. Biochemical experiments would indicate whether the interactions between Kms proteins and Plo1 occur in fission yeast and they can be quantitated for protein production patterns during meiosis.

The biological function of a Kms1-Plo1 or Kms2-Plo1 interaction needs to be assessed further. The interaction may play a role with Plo1 in meiosis. For instance, SPB localisation of Plo1 could be important for progression through meiosis, but studies have not been performed to assess this requirement. Localisation studies would indicate whether Plo1 colocalises with either Kms1 or Kms2. The prediction would be for some overlap to occur in fluorescence or antibody-tagged microscopy studies, as Plo1 does localise to the SPB and Kms1 and Kms2 are present at the SPB throughout a mitotic or throughout a meiotic cell cycle. The deletion constructs indicate that amino acids 206-607 are sufficient to interact withPlo1. Recent data indicates that amino acids 539-607 are sufficient and are required for interaction with Sad1, a known SPB component (Miki et al., 2004). This is the region where two transmembrane domains are predicted at amino acids 553-575 an 582-604 (section 2.9.b). It appears that the C-terminal region of Kms1 protein could be important for anchoring Kms1 protein to the SPB. When the C-terminal half of *kms1* is deleted in a strain, the phenotype of SPB disintegration during meiosis is observed. The same phenotype occurs when full-length *kms1* is deleted, whereas if the N-terminal half of *kms1* is deleted, the meiotic SPB remains intact (Shimanuki et al., 1997).

Whether the region of Kms1 required for SPB localisation is required for interaction with Plo1 can be assessed, by making further deletion constructs using PCR-based cloning, with restriction sites in the primers. The truncated Kms1 proteins could be assessed for interaction with Plo1 by yeast-two hybrid. If the SPB localisation region of Kms1 is not required for interaction with Plo1, it would be easier to separate issues regarding Plo1 SPB localisation and Kms1-Plo1 interaction. For instance, if the biological role of the Kms1-Plo1 interaction is for Plo1 to be recruited to the meiotic SPB, then the SPB localisation domain of Kms1 and the Plo1-binding region could be treated independently. The ideal experiment would be to disrupt the Kms1-Plo1 interaction by mutating *kms1*, such that the mutant Kms1 protein can still localise to the SPB. Within this strain, Kms1 protein would be localised at the SPB, but would not be able to interact with Plo1. Then, one could observe Plo1 localisation. To disrupt the Kms1-Plo1 interaction, a narrower binding domain could be defined using PCR-based cloning and either random mutagenesis or a systematised manner of mutagenesis, such as pentapeptide insertion, could be used. The aim of such experiments would be to create a strain that lacked the Kms1-Plo1 interaction and study the phenotype for further problems, such as progression through meiosis or problems with telomere-led bouquet formation and recombination events and separation of homologous chromosomes. It would indicate that the Kms1-Plo1 interaction is required for meiosis to occur properly and if Plo1 SPB localisation is required for progression through meiosis.

Similar experiments could be done for Kms2. Analysis using deletion constructs of Kms2 showed that amino acids 77-273 are required for interaction with Plo1 and random mutagenesis that amino acids 77-251 were sufficient for interaction with Plo1. This was determined by *lacZ* activity. There is a coiled-coil region at amino acids 170-211 within the Plo1-binding region and a putative binding site, STP, at amino acid 138 (Figure 2.9.C). The putative Plo1-binding site STP within Kms2 corresponds to a site in Kms1 in

alignments (Figure 2.10). However, this site occurs at amino acid 163 in Kms1, which is not required for Plo1 binding. When amino acids 1-205 are deleted, Kms1 still interacts with Plo1. To determine whether the putative binding site in Kms2 is required, smaller deletions or a targeted substitution using site-directed mutagenesis need to be made and assayed for interaction.

To study the Kms2-Plo1 interaction, attempts were made to disrupt the interaction. For Kms2, point mutations did not disrupt the interaction. Either a point mutation which disrupted interaction was made, but was not detected, or the mutation was not made. If the number of colonies assayed was insufficient, a mutation causing disruption of interaction could remain undetected. Amino acids 77-273 comprise 197 amino acids encoded by 591 nucleotides, which were randomly mutagenesised. Considering 3150 colonies were assayed for disrupted interactions over 591 nucleotides and given that 1/1,000 base pairs are mutated during PCR (section 3.3.b, Reynolds and Ohkura, 2001), it is unlikely that a mutation that disrupted the interaction was not detected. The other possibility is that the Kms2-Plo1 interaction was not disrupted and that it cannot be disrupted by point mutation. Further deletions could be used to identify if there are more than one region binding Plo1. It is unlikely that the putative bindingsite STP is the interacting-region, given that the corresponding site in Kms1 can be deleted without affecting interaction with Plo1. It is more likely that the coiled-coil is important for interaction. If the coiled-coil domain (amino acids 170-211) or portion of the domain were deleted, one could assay whether the coiled-coil domain in Kms2 was required for interaction.

4.7. Sid4-Plo1 interaction

4.7.a. Regions of Sid4 interacting with Plo1

The hypothesis is that Plo1 interacts with multiple proteins to achieve its many roles during the cell cycle. It appears that Plo1 interacts with Sid4 to achieve one of its roles, septation. The Sid4-Plo1 interaction found in this study and evidence in the literature (Chang and Gould, 2000; Tanaka et al., 2001) support the idea that the interaction between Plo1 and Sid4 plays a role in septation/cytokinesis. Sid4 is needed for all SIN gene products to localise to the SPB (Chang and Gould, 2000). Plo1 acts at the top of the SIN to effect septation initiation (Tanaka et al., 2001).

The advantage of two-hybrid assays in having sensitivity and the capacity to detect transient interactions are useful for finding interactors of a kinase, but these characteristics are a disadvantage as non-specific interactions can be detected indiscriminately. Recent data shows that there is a biochemical interaction between Sid4 and Plo1 by immunoprecipitation experiments (Morrell et al., 2004). From GST-pulldowns, the interaction between Sid4 and Plo1 has been shown to be direct (Morrell et al., 2004). When mutations of Sid4 were made and assessed for interaction by two-hybrid assay, amino acids 155-570 were sufficient for interaction with Plo1 (Figure 3.8). Deleting the first 152 amino acids did decrease *lacZ* activity (Figure 3.8), but amino acids 1-153 were not sufficient for interaction with Plo1 (Figure 3.8). In addition, premature termination codons and frameshift mutations showed that amino acids 1-311 are not sufficient for interaction (Figure 3.8). In a different study, amino acids 1-461 of Sid4 are sufficient for interaction with Plo1 by yeast two-hybrid assay (Morrell et al., 2004), in agreement with this study. Contrary to the two-hybrid data gathered here from random mutagenesis indicating that amino acids 1-311 are not sufficient, amino acids 1-191 of GST-tagged Sid4 were sufficient to interact with Plo1 (Morrell et al., 2004). The reason for disparity may be due to the different types of experiments. Whether there is a protein in bacteria that can mediate interaction between Sid4 and Plo1, such as Cdc11 or a protein that behaves similarly to Cdc11, could account for the difference between two-hybrid and GST-

pulldown data. Using *in vitro* transcription-translation would provide a different type of experiment that might resolve this issue. The other aspect is that in the two-hybrid system, perhaps dimerisation of Sid4 facilitates interaction with Plo1, whereas in the *in vitro* system of using bacterially produced proteins, there may be a higher concentration of Sid4 that is exposed for interaction with Plo1.

The two-hybrid data generated in this study would indicate that amino acids 311-570 are required for interaction with Plo1. This region contains two coiled-coil domains and one putative binding site. Amino acids 301-490, most of which is covered by 311-570, is required for dimerisation of Sid4. Ideally, deletion constructs should be made using PCR-based methods to determine whether amino acids 1-191, 155-311 and 311-570 are sufficient for interaction with Plo1 and assessed for interaction by two-hybrid assay and GST-pulldown experiments. This would help to resolve conflicting data concerning the Plo1-binding regions of Sid4. Amino acids 1-191 of GST-Sid4 that is sufficient to interact with Plo1 (Morrell et al., 2004) contains the Cdc11-interacting region of Sid4 (amino acids 21-152) (Tomlin et al., 2002). From this study, two-hybrid data indicate that the first 152 amino acids of Sid4 are not required for interaction with Plo1. Two possibilities exist: the Cdc11-interacting domain of Sid4 is dispensable for interaction with Plo1 or the dimerisation regions is not required for interaction with Plo1. The SPB localisation domain of Sid4 is not required for interaction and no conflict in data occurs over this, as two-hybrid data here and in Morrell et al. show that 571-660 or 462-660 can be deleted respectively, while maintaining interaction with Plo1 (Morell et al., 2004).

4.7.b. Disruption of Sid4-Plo1 interaction in fission yeast

The aim to produce mutant proteins that were specifically disrupted in interaction with Plo1, was performed by randomly mutagenising *sid4*. The intent of creating such a mutant

was to study the effect of disrupting the interaction in fission yeast. The two single point mutants of Sid4, S158L and V264I, that no longer interacted with Plo1 were made. Both mutants were able to rescue the *sid4-SA1*^{ts} strain at the non-permissive temperature, just as wild-type Sid4 does (Results Chapter 4). The *sid4-SA1*^{ts} strain transformed with empty vector, the negative control, did not support growth at the non-permissive temperature. However, the mutants of Sid4 protein, S158L and V264I, are expressed at lower levels in comparison to wild-type Sid4 in the same plasmid. Therefore, the apparent weakened interaction is probably due to decreased Sid4 protein levels, not an actual disruption. It would be difficult to assess the interaction with Plo1 of the mutant Sid4 proteins compared to wild-type by immunoprecipitation, due to a 10-fold difference in protein levels (Figure 3.12).

The single mutants of Sid4 (S158L and V264I) no longer interact with Plo1, but can rescue the temperature-sensitive strain of *sid4-SA1* encoding a mutation, L629P. Sid4 S158L and Sid4 V264I may be able to localise to the SPB, as the SPB localisation domain is from amino acids 570 to 660 (Okazaki et al., 2002). The endogenous Sid4 protein found in the *sid4-SA1* temperature sensitive strain has a single mutation in Sid4 protein altering a leucine to a proline, L629P. Sid4 L629P would most likely interact with Plo1, as the minimal binding domain for Plo1 consists of amino acids 155-570 (this study) or 1-191 (Morrell et al., 2004). The single mutants (S158L and V264I) may heterodimerise with the temperature-sensitive strain's endogenous Sid4L629P. My data indicate that S158L and V264I are capable of dimerising with wild-type Sid4 by yeast two-hybrid assay. If the single mutants of Sid4 (S158L and V264I) do form heterodimers with the temperature sensitive strain's endogenous Sid4 L629P, then the components of the SIN would be able to be localised to the SPB and Plo1 could interact with its necessary mediator. Thus, the Sid4 protein mutants, S158L or V264I, and L629P, would compensate for one another. Experiments that would test whether the single mutants S158L and V264I could rescue a

sid4 null strain would clarify why the single mutants may be able to rescue the *sid4-SA1*^{ts} strain (in which the mutation is L629P in Sid4).

The Plo1-binding regions must be identified more specifically by both two-hybrid assays and a biochemical assay. Then a narrower binding domain could be defined. To continue with the approach used in this study, the narrower Plo1-binding domain of Sid4 could be randomly mutagenised or specifically targeted for mutation and then assayed for interaction by two-hybrid analysis in budding yeast. It would be better to be able to assess the level of protein in budding yeast and to have a tag within the prey vector. If the interaction was decreased and the protein levels roughly the same as wild-type then, the *sid4* mutant genes are expressing protein that have less affinity for Plo1. Then the *sid4* mutants and wild-type *sid4* would need to be cloned into fission yeast vectors. The protein levels would need to be assessed in fission yeast. and the interaction would need to be assessed by immunoprecipitation. Experiments to assay whether the non-interacting Sid4 mutant proteins could rescue *sid4-SA1*^{ts} and *sid4*Δ could then be performed. In this manner, one could assess the effect of disrupting the interaction on fission yeast; whether the interaction is essential or if septation defects are observed.

New data from Miki et al. (2004) shows that Kms1 interacts with Sid4 by yeast two-hybrid. Considering the role Kms1 plays during meiosis, it is possible that Sid4 plays a role in meiosis. It would be useful to observe synchronised fission yeast undergoing meiosis for Sid4 and Plo1 localisation. Visualising colocalisation of Sid4 and Plo1 even in a vegetative state would give more information for the physiological role of an interaction. It is possible that Sid4, Kms1 and Kms2 interact to recruit Plo1 to the SPB during meiosis.

Considering that biochemical experiments have been done, albeit by another group, supporting the Sid4-Plo1 interaction identified by two-hybrid screening, genetic

experiments could help to identify what role the interaction plays. Components of the SIN pathway were found as high *plo1*-dependent mutants (Cullen et al., 2000). *cdc11*, *sid2*, *cdc7* and *spg1* were found to have mutations that did not support growth at higher temperatures except in the presence of high levels of Plo1 (Cullen et al., 2000). Experiments assessing if high levels of Plo1 could rescue the *sid4-SA1*^{ts} strain would indicate whether Plo1 could activate septation. To dissect out the SIN pathway, a Plo1 substrate can be searched for. Given that *cdc11*, *spg1*, *cdc7* and *sid2* are high Plo1-dependent mutants, the mutations within these genes could give be assessed for putative binding and phosphorylation consensus sequences.

Within the *sid4-SA1*^{ts} strain, Sid4 localisation has not been examined. From other data, deletion of the C-terminal 46 amino acids results in loss of SPB localisation of Sid4 (Tomlin et al., 2002). To examine if Plo1 overproduction could rescue *sid4-SA1*^{ts} at the non-permissive temperature could give information on the biological role of the interaction, particularly to search for interactions with other proteins, this strain could be tested for viability at the non-permissive temperature. Excess levels of Plo1 may be able to interact with other components of the SIN. However, in the *sid4-SA1*^{ts} strain, none of the SIN components would be concentrated at the SPB. Another type of genetic experiment that might be predicted to be more successful would be to overproduce Plo1 in a *sid4*Δ strain carrying Sid4 that no longer interacts with Plo1, but could localise to the SPB and interact with Cdc11. In this strain, the SIN components would be predicted to be localised at the SPB. If Plo1 overproduction was able to rescue a *sid4* mutant strain, which produced SPB-localised Sid4 that did not interact with Plo1, but did interact with Cdc11, the septation phenotype could be observed and compared with that of wild-type or other types of *sid4* mutants. Thus, overproduction of Plo1 could suppress a mutant phenotype. In this experiment, Plo1-interacting proteins could be searched for. Another type of experiment would be to overproduce a SIN component downstream of the Sid4-Plo1

interaction. For instance, overproducing Sid2 would most likely overcome a disrupted Sid4-Plo1 interaction. It would be useful to observe Plo1 localisation, particularly for movement from the SPB to the midzone in fission yeast. Sid2 moves to the SPB in fission yeast from the SPB (Simanis, 2003a; Simanis, 2003b; Sparks et al., 1999). Whether Plo1 needs to translocate to the midzone for cytokinesis to take place as Plk1 is required to in human cells (Neef et al., 2003) would be an interesting study. Analysing the other components, particularly Cdc7 and Spg1, would be very useful. Cdc7 SPB localisation follows peak kinase activity of Plo1 and septation follows SPB localisation of Plo1 (Mulvihill et al., 1999; Tanaka et al., 2001). One last type of genetic experiment would be to assay for synthetic lethality, by combining the *sid4*^{ts} strain with a *plo1* mutation. However, at the time of this study, the *plo1* point mutants were not conditional; they were lethal (Reynolds and Ohkura, 2003). Point mutations and deletion mutants of *plo1* could not rescue a *plo1*Δ strain, although data from another recent study indicate that *plo1-ts2* could survive. *plo1-ts2* has an amino acid substitution, E139K and the mutant cells have difficulties entering mitosis. In *plo1-ts2 cut12.s11*, Plo1 is not recruited to the SPB during interphase. Combining *plo1-ts2* with *sid4-SA1*^{ts} and observing Plo1 localisation could be useful. The double mutant *plo1-ts2 sid4-SA1*^{ts} may not survive at the permissive temperature due to difficulties entering mitosis in combination with a lack of septation. If it does survive, one might observe if Plo1 localises to the SPB even during mitosis.

Ultimately, more experiments need to be performed to assess the function of Sid4-Plo1 interaction. The two-hybrid assay is only one type of genetic experiment. Overproduction of Plo1 and resulting suppression of *sid4* mutant phenotypes would probably strengthen the cause for a biologically relevant interaction, as would synthetic lethality. Other than genetic experiments, biochemical studies have been performed by others, but this should be reproduced to gain a clearer result of which region of Sid4 interacts with Plo1. Localisation studies can be used to assess the cell cycle dependence of the interaction. The

evidence points to the Sid4-Plo1 interaction being required for septation. Finally, whether each SPB, Kms1, Kms2 and Sid4, interacts with the other needs to be assessed by two-hybrid assays and biochemical studies. A new role for Plo1 during meiosis may exist.

Chapter 5. Materials and Methods

5.1. Materials

5.1.a. Chemicals

Chemicals used were from Sigma, BDH and Melford laboratories, unless otherwise stated.

5.1.b. Solutions

Table 5.1. Commonly used solutions.

Solution	Composition
10xPBS	NaCl 0.8%(w/v) OR 135mM KCl 0.02%(w/v) OR 2.5mM Na_2HPO_4 0.144% OR 10mM KH_2PO_4 0.024% OR 1.8mM
50xTAE	2M Tris 0.05M EDTA (pH8) 5.7% (v/v) glacial acetic acid
10xTE	100mM Tris-HCl, pH7.5 10mM EDTA
Sample buffer for protein	50mM Tris-Cl pH6.8 300mM dithiothreitol 6% SDS 0.3% bromophenol blue 30% glycerol
DNA loading dye	0.25% bromophenol blue 0.25% xylene cyanol 15% Ficoll

5.1.c. Molecular biology reagents

Agarose (InVitrogen-ultrapure) was used for gel electrophoresis of DNA, acrylamide/bis acrylamide 30% (Severn Biotech Ltd.Worcester,UK) for gel electrophoresis of proteins. 1 kb ladder from NEB or Promega was used. Restriction enzymes were from NEB, or in some cases Roche (formerly Boehringer-Mannheim). Klenow, T4 DNA ligase, shrimp

alkaline phosphatase (SAP) and Exonuclease I (ExoI) were obtained from NEB. Tween was diluted in PBS for washes of nitrocellulose blots containing protein. Antibodies were diluted in PBS containing 0.1% Tween and 3% skim milk powder. Enhanced chemiluminescence (ECL) solutions were purchased from Amersham. Taq was procured from Roche and Taq/Pwo from Hybaid (no longer available). dNTPs were from Boehringer Mannheim.

5.1.d. Media

Bacto-agar (Difco), Bacto-tryptone (Difco), yeast nitrogen base (Difco), and yeast extract (Difco) was used to make medium. Adenine, leucine, histidine and tryptophan were from Sigma. 10,000x Minerals was made up in 200ml of distilled water of 1g H_3BO_3, 1.04g $MnSO_4.4H_2O$, 0.8g $ZnSO_4.7H_2O$, 0.4g $FeCl_3.6H2O$, 288mg H_2MoO_4, 80mg H_2MoO_4, 80mg $CuSO_4.5H_2O$, 2g citric acid and 20mg KI. 1,000x Vitamins was made up in 500ml of distilled water of 5g inositol, 5g nicotinic acid, 0.5g D-pantothenic acid and 5mg D-biotin.

5.1.e. Antibodies

Antibodies used in this study include, mouse anti-HA 12CA5 used at 1:1000 (Roche), rabbit anti-Plo1 antibody HN184 used at 1:500 (Ohkura et al., 1995) and mouse anti-Gal4 activation domain antibody incubated at a concentration of 1:600 (Clontech). Goat anti-mouse and goat anti-rabbit antibodies conjugated with horseradish peroxidase (Jackson laboratories) were used at 1:1000 for detection by chemiluminescence.

Table 5.2. Media.

Medium	Composition
Luria Broth	1% w/v bacto-tryptone 0.5% w/v yeast extract 0.5% w/v NaCl pH adjusted to 7.2 with 10N NaOH
SOC	2% w/v bacto-tryptone 0.5% w/v yeast extract 0.05% w/v NaCl 2.5mMKCl/ pH adjusted to 7.0 with 10N NaOH after autoclaving added 10mM $MgCl_2$ 20mM glucose
YPDA	1% w/v yeast extract 2% w/v bacto-peptone 2% w/v glucose 0.003%w/v adenine sulfate
EMM	2% w/v glucose 0.3% w/v KH phthalate 0.18% w/v Na_2HPO_4 0.5% w/v NH_4Cl 0.01% w/vNa_2SO_4 0.1% w/v MgCl2 0.1% v/v 1,000x Vitamins 0.01% v/v 10,000x Minerals
YE	0.5%(w/v) Difco yeast extract 3% (w/v) glucose 0.02% (w/v) adenine 0.02%(w/v) uracil
MB	0.5% w/v Glucose 0.05% w/v KH_2PO_4 0.05% w/v $MgSO_4.7H_2O$ 0.01% w/v NaCl 0.01% $CaCl_2.2H_2O$ 0.5% w/v $(NH_4)_2SO_4$ 0.036%w/v KCH_2COOH 0.1% v/v 1,000x Vitamins 0.01% v/v 10,000x Minerals
SPA	1% Glucose 0.1% w/v KH_2PO_4 0.1% v/v 1,000x Vitamins 0.01% v/v 10,000x Minerals

5.1.f. Plasmids used in this study

Table 5.3. Plasmids.

Plasmids	Description
pGBT9 (lab stock)[1]	Gal4 DNA binding domain with multiple cloning site (MCS)‡ allowing bait cDNA to be cloned to form a N-terminal fusion protein under the control of a truncated *ADH1** promoter with *TRP1* as a selective marker.
pGBT9*plo1*[2]	Full-length plo1 cDNA cloned into the BamHI site of bait plasmid pGBT9.
pGAD424 (lab stock)[1]	Gal4 DNA activation domain with MCS allowing prey cDNA to be cloned to form a N-terminal fusion protein under the control of a truncated *ADH1** promoter with *LEU2* as a selective marker.
cDNA library in pTNTH7[3]	The meiotic cDNA library was made by having SalI at the 5'-site and NotI at the 3'-site. The PstI site is lost in this plasmid.
pGADGH[1]	Gal4 DNA activation domain with MCS allowing prey cDNA to be cloned to form a C-terminal fusion protein under the control of full-length *ADH1** promoter with *LEU2* as a selective marker.
pAA100 pREP1xHA[4]	Three HA repeats with MCS allowing gene of interest to be cloned to form a C-terminal fusion protein under the control of the nmt1 promoter with *LEU2* as a selective marker.
pAA102 (this study)	Full-length Sid4 in pAA100
pAA104 (this study)	Full-length S158L Sid4 in pAA100
pAA106 (this study)	Full-length Sid4 in pGBT9
pAA107 (this study)	Full-length V264I Sid4 in pAA100

*-*ADH1* is the alcohol dehydrogenase gene of *S. cerevisiae*.
‡(-multiple cloning site (MCS) is a region within the plasmid that has been constructed to have many restriction enzyme sites
1-Originally from Clontech, but no longer commercially available, 2-(Reynolds and Ohkura, 2003), 3-Gift of Dr. C. Shimoda, 4-gift of Dr. Hiroyuki Tanaka

5.1.g. Yeast strains used

Table 5.4. Strains of yeast.

Strain	Genotype
S. cerevisiae	
Y190	*MAT*a *ura3-52 leu2-3,112 his3-200 trp1-901 ade2-101 lys2-801* Δ*gal*4 Δ*gal*80, *cyh*'2 *LYS2::GAL1* $_{UAS}$*-HIS3 TATA - HIS3,URA3 : : GAL1 UAS -GAL1 TATA -lacZ*
Y190+pGBT9*plo1*	Y190 transformed with pGBT9plo1, bait plasmid
S. pombe	
sid4-SA1	*h*$^{-}$ *leu1-32 ura4D18 sid4-SA1*^{ts} *ade6-210*

5.1.h. Bacterial strains used

Table 5.5. Strains of bacteria.

Bacteria	Genotype
XL1 Blue (Stratagene)	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 lac [F' *pro*AB laq^{lq}ZDM15 Tn10 (Tet^{r})]
JM109 (Promega)	*sup*E44 *hsd*R17 (r_k-, m_k+) *rec*A1 *end*A1 *gyr*A96 *thi rel*A1 Δ(lac-*pro*AB), [F' *tra*D36 *pro*AB laq^{lq}ZDM15]
Epicurian coli (Stratagene) XL2 Blue MRF'	Δ (*mcr*A)183 Δ (*mcr*CB-*hsd*SMR-mr)173 *sup*E44 *end*A1 *rec*A1 *gyr*A96 *thi rel*A lac [F' *pro*AB laq^{lq}ZΔM15 Tn10 (Tet^{r}) Amy Cam']^{a}

5.1.i. Oligonucleotides used

Table 5.6. Oligonucleotides used in this study.

Oligo number	Oligo name	Sequence
NR104	GADSEQ	gataccccaccaaacccaaaaaaagag
NR113	GADF	aataccactacaatggatgatgta
NR114	GADR	gtgaacttgcggggtttttcagtatc
AA002	GADSEQ early	actgtcacctggttggac
AA004	Kms2 Forward	ttgacgcattgcgacttaat
AA005	Kms2 Reverse	tcaagttctgatgccagtaa
AA006	Kms1 Forward	gtctcaatttaccgacagtg
AA007	Kms1 Reverse in pGAD424	gatcagaggttacatggcca
AA008	Sid4 Reverse	agtctacgaccttgttgcag
AA009	Kms2 Internal Forward	gaggcgcttttatggagtca
AA010a	Sid4 Internal Forward	gacatcatgagccttcgttt
AA010b	Sid4 Internal Reverse	ctgtagacccgtcattcagc
AA011	Sid4 Middle Forward	tttacaggttccaccaagcc
AA012	Kms1 Internal Forward1	gtaagtttacagatcagact
AA014	Kms1 Internal Forward2	ctctaaccttgacgaaatgg
AA017	Cdc11 Forward for gap-repair into pGBT9	aataagtgcgacatcatcatcggaagagagtagt aacaaaggtcaaagacagttgactgtatcgccgg aattcccggggatggaacagttatggcttga
AA018	Cdc11 Reverse (pGBT9) for gap-repair into pGBT9	ttttttttataacttatttaataataaaaatcataaatca taagaaattcgcccggaattagcttctgcagctcg agtctagttggatgatttggtga
AA019	Sad1 Forward for gap-repair into pGBT9	aataagtgcgacatcatcatcggaagagagtag taacaaaggtcaaagacagttgactgtatcgccg gaattcccggggatgtttactaatacaccggt
AA020	Sad1 Reverse for gap-repair into pGBT9	ttttttttataacttatttaataataaaaatcataaatc ataagaaattcgcccggaattagcttctgcagct cgagtttaagatgaatcttgacccg
AA022	Cdc11 Forward	cgtcacagatagattggctt
A034	pREP1-Nde1-Sid4 Forward	taaatcatatggatgaggcttttggtga
AA043	Sid4 pREP Reverse	gtatgggtagcggccgcgacacaaact acgttttttaagctcccttatc

5.2. Methods

5.2.a. Amplifying cDNA library in bacteria

Firstly, the meiotic cDNA library pTNTH7 was amplified in supercompetent Epicurian coli (Stratagene) using 100 ng of DNA. The meiotic cDNA library was a kind gift from Dr. Shimoda. b-mercaptoethanol was added to give a final concentration of 25 mM and the cells were incubated on ice for 30 minutes, heat-shocked at 42°C for 30 seconds and then incubated with SOC medium for bacteria (Table 5.2). The incubation lasted for 1 hour at 37°C and was left shaking at 225-250rpm (rotations per minute). The cells were centrifuged, the supernatant was decanted and cells were resuspended in 3 ml of water. 3 ml of cell suspension was plated on regular LB-Amp plates of 45mm radius to determine the transforming efficiency. The remainder of the cells were plated on large 245mm square plates (Corning) and incubated at 37°C overnight. 5 ml of water was added to both plates and the plates were scraped of cells. 5ml of 60% glycerol was added to bring the total volume to 10 ml and multiple aliquots were frozen at –80°C. 1 ml of cells was thawed and used to inoculate 1 litre of Luria-Broth to be grown overnight at 37°C. 500 ml of cells were collected by centrifugation, the cell pellet was frozen down at –20°C for later use. The other 500 ml was used to make 4 maxipreps (QIAgen) of the cDNA library pTNTH7.

5.2.b. Transforming yeast containing bait plasmid with cDNA library

150-200 µl of the cDNA library was used to transform the *Saccharomyces cerevisiae* strain Y190 + pGBT9plo1. The yeast was inoculated into 100ml of minimal medium with histidine (H), adenine (A) and leucine (L), referred to as SD + HAL. Yeast in liquid medium was grown overnight and diluted to 500ml the following day. After 3 hours and 45 minutes of growth, the cells were collected by centrifugation, washed with distilled water (dH_2O) and resuspended in 4 ml of 0.1M LiAc in TE. 200 µl of cDNA, 1 ml salmon sperm DNA (10 mg/ml) and 30 ml of 50% PEG in 0.1M LiAc/1xTE were added to the cells. The cell suspension was incubated at 30°C for 30 minutes, then 3.4 ml DMSO was added and the yeast were heat-shocked at 42°C for 15 minutes, immediately chilled on ice

and then resuspended in 7ml of dH_2O. Two dilutions of 1/10 and 1/100 were plated on SD + HA. During the first transformation, 100ml of the transformed yeast was used for one plate. The total amount of transformed yeast was plated on SD containing adenine and 10mM, 20mM, 30mM or 40mM 3-aminotriazole (3-AT).

After 3 days at 32°C, large colonies were picked and patched onto SD containing adenine and 10mM 3-AT and grown at 32°C for one day. These were replica-plated onto SD containing adenine and 10mM 3-AT and an X-galactose overlay assay was performed on the original plate. The colonies on the replica plate corresponding to blue colonies on the original plate were considered positive for a bait-prey interaction. Single colonies were isolated by streaking cells out and allowing them to grow. Then, at least six colonies were retested and subjected to X-galactose assay. For those that remained positive, colony PCR was performed from fresh colonies.

5.2.c. X-galactose overlay assay

10 ml of agar containing X-galactose was used as an overlay on plates. Agar was used at 5% w/v in a 10ml overlay containing 0.27 M K_2HPO_4 and 0.17 M KH_2PO_4, 0.1% SDS and 0.22% X-galactose, which was dissolved in N,N-dimethylformamide (Sigma).

5.2.d. Single colony isolation

Cells were streaked out from the original patch and several single colonies were isolated, patched and replica-plated and subjected to the X-galactose overlay assay. In addition, YE (yeast extract medium, Table 5.2) glycerol stocks were made of positive clones.

5.2.e. Colony PCR and sequencing

Cells from a freshly grown colony of *S. cerevisiae* containing both bait vector and cDNA of interest were picked with a yellow tip and resuspended in 10 ml of 0.02 N NaOH, freshly made from 10N NaOH. Each tube was vortexed to resuspend cells and heated at 100°C for 5 minutes, immediately chilled on ice and spun down briefly. Then the cells were vortexed again to mix the cells well and 2 µl of cell suspension was used as template for PCR reactions. 50 ng of each primer (oligos GADF and GADR, NR113 and 114 see Table 7.6) were used in a 32 ml reaction with 1 pmol/µl dNTPs in 1X PCR buffer. Just prior to putting the 0.5ml PCR tubes in a preheated PCR machine, 0.5 µl of Taq polymerase (Boehringer Mannheim) was added. The PCR program was set as follows: 94°C for 3 minutes x1 cycle, 94 °C 30s, 55 °C 90s, 72 °C 3 minutes x 31 cycles and a finishing step at 72 °C for 5 minutes. 15 ml of the PCR product was run on a 0.7% agarose gel containing 0.5 µg/ml ethidium bromide to determine the size of the cDNA insert for each positive clone. The remaining portion of the colony PCR was used for sequencing reactions. 6 µl of PCR product was transferred such that no cell debris was pipetted and 1 µl of Exonuclease I (Amersham 10 U/µl) and 1 µl of shrimp alkaline phosphatase (USB 1U/ µl) were added. The PCR product was incubated with ExoI and SAP at 37°C for 15 minutes for excess primer to be degraded and 5'phosphates to be cleaved. Then, the enzymes were inactivated at 80°C for 15 minutes. Following this 4 µl of oligo GADSEQ (NR104) was added with 8ml of sequencing mix and PCR was performed as such: 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes x 25 cycles. 2 µl of 3M NaAc pH6 were added to the 20 µl reaction and 50 µl of 100% ethanol. The samples were left at -80°C for 20 minutes and were spun down for two minutes, then rinsed with 100 ml of 70% ethanol, spun down and left to air dry. These were then given to the facility at the Institute of Cell and Molecular Biology for automated sequencing.

5.2.f. Selectively losing the bait plasmid

When recovering plasmids from yeast, the bait plasmid was isolated more frequently than the prey plasmid. To recover plasmids that contained the cDNA of a positive interactor more efficiently, the *TRP1*-carrying bait plasmid was lost from yeast, so that cells would contain only one plasmid rather than two. By growing with the nutrient tryptophan, which corresponds to the auxotrophic marker of the bait plasmid, the chance for yeast to lose the bait plasmid was increased. Cells were streaked from the glycerol stocks onto SD + HAW. After waking up the positive clones, the strains were grown in 5ml liquid SD + HAW overnight and then plated at 500 cells per plate so that single colonies could easily be replica-plated. After growing for three days, cells were replica-plated onto SD + HA and SD + HAW. Those colonies that died on SD + HA, the corresponding colonies living on SD + HAW lost the *TRP1*-carrying bait plasmid. These colonies were picked and inoculated into 5 ml liquid SD + HAW. The next day they were subjected to a procedure to rescue the prey plasmids.

5.2.g. Rescuing the prey plasmid

Cells were centrifuged and resuspended in 200ml of extraction buffer consisting of 2% Triton X100, 1% SDS, 100 mM NaCl, 10 mM Tris.HCl pH 8.0 and 1 mM EDTA pH8.0. 400 ml of acid-washed glass beads (Sigma) were added as well as 200 ml of phenol/chloroform/isoamyl alcohol mix (25:24:1) in a 2 ml screw-cap tube. The cell mixture was subjected to 20 minutes of vigorous shaking using a vortex machine. Cells were checked microscopically for breakage. Broken cells appeared as dark circular shadows. Tubes were centrifuged at top speed for 10 minutes and 140 ml of supernatant was transferred to a clean 1.5ml tube. 500 µl of ethanol/ammonium acetate mix (6:1 7.5M NH_4Ac) was added; each tube was vortexed well and spun at top speed for 20 minutes.

Pellets were washed with 250 µl of 70% ethanol, air-dried and resuspended in 10 µl of water.

3-5µl of this DNA preparation was transformed use to transform either XL1Blue competent cells or JM109 high efficiency competent cells (Promega) and plated on LB-Amp. Minipreps of plasmid DNA were prepared and NotI restriction digests to make certain they were prey plasmids. Bait was not cut with NotI, while prey was linearised. HindIII digests were also performed to differentiate between bait and prey plasmid (see Results Figure 2.5).

5.2.h. Retransforming Y190

The plasmids that were prey were then transformed back into Y190 along with bait plasmid (pGBT9*plo1*) or empty bait vector (pGBT9). The same proportion of cells were used as in the first protocol describing yeast transformation (section 5.2.b) so that cells were resuspended in a volume of 0.1MLiAc/TE corresponding to the volume of DNA to be transformed. For instance, 100 µl of cells in LiAc/TE would be transformed with 5 ml prey and 5 ml bait or empty vector, 20 µl ssDNA and 750 µl of PEG/LiAc/TE. 85 µl of DMSO was added prior to heat shock and the cells were plated on SD + HA. After three days, 4 colonies from each plate were patched onto SD + HA and an X-gal overlay assay was performed on the patches. In addition, the plasmids were sequenced to make certain they matched the colony PCR product's sequence. This was done using 2.8ml DNA, 2 µl oligonucleotide, 4 µl reaction mix in a 10 µl reaction.

5.2.i. Constructing deletions and point mutants of *sid4*, *kms1* and *kms2* cDNAs

Restriction enzymes from New England Biolabs were used for cloning. Single cutters were identified and used in combination with upstream restriction enzymes, EcoRI, BamHI, SalI or the downstream restriction enzyme NotI. The deletion constructs for Sid4 are described here in detail. The simplest option would be to cut with NotI and make a 3'-deletion, but the cDNA library was made with NotI, because NotI does not occur frequently in the fission yeast genome. None of the cDNAs have NotI sites. Thus, three other options were used to delete a region of the gene and perform intramolecular ligations . For a 3'-deletion frame of the nucleotide series did not have to be taken into account, so NotI in combination with another restriction enzyme and subsequent modification by Klenow and ligation was sufficient. For 5'-deletions, the frame of nucleotides needed to be considered. Enzymes found in pGAD424, such as EcoRI, BamHI and SalI were used on their own or in combination with enzymes that resulted in compatible ends after digestion, such as BglII and XhoI for BamHI and SalI respectively. No modification was required afterwards and ligation could be performed. The last option for intramolecular ligations was to make a 5-deletion using enzymes found in the vector in combination with any restriction site found in the cDNA. In this case Klenow modification was necessary prior to intramolecular ligation.

For random mutagenesis the procedure was repeated and the plasmid was digested on either side of the minimal binding domain. The PCR product corresponded to the plasmid gene of interest and extended 100 base pairs on either side of the 5'- and 3'-cuts, so that in budding yeast the gapped, linearised plasmid could be repaired with individual PCR products. The PCR program for random mutagenesis was set as follows: 94°C for 3 minutes x1 cycle, 94 °C for 30 seconds, 55 °C for 90 seconds, 72°C for 3 minutes x 25 cycles and a finishing step at 72 °C for 10 minutes.

5.2.j. Gel electrophoresis of DNA:

0.7% agarose gels were made up in TAE in a volume of 100ml with 0.2-0.5 μg/ml ethidium bromide. The agarose gels were run at 100V (max. 5V/cm) for 30 minutes to 1 hour and for analytic gels, occasionally at 10V overnight.

5.2.k. Isolation of DNA from agarose gel

PCR products were run on a gel and the band of appropriate size cut out. The linearised plasmid was also run on a gel and the band cut out. The pieces of gel containing DNA were purified using the QIAgen gel extraction kit, according to the manufacturer's instructions. A volume of 30 μl was eluted and 3-5 μl of linearised plasmid with 7-12 μl of gel-purified PCR product were used for ligation reactions using 1 μl of T4 DNA ligase (NEB) in a final volume of 10-15 μl. The ligation mixture was incubated at 14°C overnight, or over a weekend. Commercially available competent cells (JM109, Promega) were transformed with the ligation mixture.

5.2.l. Bacterial transformation

JM109 cells were incubated on ice for five minutes, gently mixing them periodically. 50, 66 or 100 μl of cells were aliquotted into prechilled tubes. 3-5 μl of plasmid extracted from yeast, or the whole volume of a ligation mixture was used to transform cells, as long as the volume of DNA did not exceed 10% of the aliquot volume. The DNA and cells were incubated for ten minutes on ice, mixing periodically. The cells were heat-shocked in a 42°C waterbath for 45-50 seconds and immediately placed on ice for two minutes. Cold SOC was added to the DNA/cell mixture; 900 μl of SOC for a 100 μl aliquot of cells, 600 μl for 66 μl, and 450 μl for 50 μl. After medium was added, the cell suspension was

shaken in a water bath for one hour at 37°C. 1/10 dilution and an undiluted aliquot of 100µl were plated on LB-Amp plates.

5.2.m. Western blotting for Gal4AD and HA

Yeast strain Y190 containing pGBT9-*plo1*$^+$ and pGAD424-*sid4*$^+$ was grown overnight in liquid medium to ~ $6x10^7$ cells/ml. $2x10^7$ cells were collected and broken open with 1.85M sodium hydroxide and 7.5% β-mercaptoethanol. Protein was extracted by incubation on ice, treatment with 55% trichloroacetic acid and centrifugation. The supernatant was resuspended in 300ml of sample loading buffer and 10-15 µl was used for loading protein gels. A 10% separating polyacrylamide gel was used for electrophoresis of protein, and once transferred, a 1/600 dilution of mouse monoclonal Gal4AD antibody was used for blotting the membrane containing transferred proteins. A 1/2,000 dilution of goat anti-mouse secondary antibody conjugated with horseradish peroxidase (HRP) was used for detecting the primary antibody. Enhanced chemiluminescence was used to detect the protein by exposing the substrate to HRP and X-ray film.

For protein extracts from *Schizosaccharomyces pombe sid4-SA1*ts with *nmt1-sid4* wild-type or mutant (S158L, V264I), 100ml of cells were grown overnight to 10^8 ($4-5x10^6$ cells/ml). Fresh extraction buffer was made of 0.1M TrisHCl, pH8 and 1mM PMSF. The 100 mM stock of PMSF was kept in ethanol at –20°C. 200 µl of extraction buffer was used to resuspend the pellet of 10^8 cells. The cell mixture was transferred to a 2 ml screw-cap tube and shaken intensely for 20 seconds (Hybaid Ribolyser) at a speed of 4. The cells were examined microscopically and up to three more 20 second intervals of intense shaking were performed until 50% of cells were broken. The extract was transferred without spinning down using a 200 µl yellow tip, as not to take up any glass beads. Usually 150 µl of ribolysed cell extract was used and transferred, such that 75 µl of 3xSDS sample buffer

was added. A final concentration of 5% β-mercaptoethanol was added and a hole was made at the top of the tube with a needle. Samples were boiled for three minutes at 100°C and 10 µl was loaded on a 10% polyacrylamide gel. After running the gel and transferring it to a nitrocellulose membrane, the latter was incubated with either 1/500 HN184 (Plo1 antibody) or 1/1,000 12CA5 (HA antibody) diluted in PBS with 0.1% Tween and 3% milk. The blots were washed with PBS-Tween. The secondary, anti-rabbit antibody for Plo1 was used at a dilution of 1/1,000 and anti-mouse antibody was used at 1/2,000. The blot was washed, then subjected to ECL and autoradiography.

References

Abrieu, A., Brassac, T., Galas, S., Fisher, D., Labbe, J.C. and Doree, M. (1998) The Polo-like kinase Plx1 is a component of the MPF amplification loop at the G2/M-phase transition of the cell cycle in *Xenopus* eggs. *J Cell Sci*, **111**, 1751-1757.

Adams, R.R., Tavares, A.A., Salzberg, A., Bellen, H.J. and Glover, D.M. (1998) pavarotti encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis. *Genes Dev*, **12**, 1483-1494.

Alberts, B., Raff, M. and Watson, J. (2001) *Molecular Biology of the Cell*. Garland Publishing, New York.

Alexandru, G., Uhlmann, F., Mechtler, K., Poupart, M. and Nasmyth, K. (2001) Phosphorylation of the cohesin subunit scc1 by polo/cdc5 kinase regulates sister chromatid separation in yeast. *Cell*, **105**, 459-472.

Alvarez, B., Martinez, A.C., Burgering, B.M. and Carrera, A.C. (2001) Forkhead transcription factors contribute to execution of the mitotic programme in mammals. *Nature*, **413**, 744-747.

Ault, J.G. and Rieder, C.L. (1994) Centrosome and kinetochore movement during mitosis. *Curr Opin Cell Biol*, **6**, 41-49.

Aumais, J.P., Williams, S.N., Luo, W., Nishino, M., Caldwell, K.A., Caldwell, G.A., Lin, S.H. and Yu-Lee, L.Y. (2003) Role for NudC, a dynein-associated nuclear movement protein, in mitosis and cytokinesis. *J Cell Sci*, **116**, 1991-2003.

Bähler, J., Steever, A.B., Wheatley, S., Wang, Y., Pringle, J.R., Gould, K.L. and McCollum, D. (1998a) Role of polo kinase and Mid1p in determining the site of cell division in fission yeast. *J Cell Biol*, **143**, 1603-1616.

Bähler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A.R., Steever, A.B., Wach, A., Philippsen, P. and Pringle, J.R. (1998b) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast*, **14**, 943-951.

Balasubramanian, M.K., McCollum, D., Chang, L., Wong, K.C., Naqvi, N.I., He, X., Sazer, S. and Gould, K.L. (1998) Isolation and characterization of new fission yeast cytokinesis mutants. *Genetics*, **149**, 1265-1275.

Barbet, N., Muriel, W.J. and Carr, A.M. (1992) Versatile shuttle vectors and genomic libraries for use with *Schizosaccharomyces pombe*. *Gene*, **114**, 59-66.

Bardin, A.J. and Amon, A. (2001) Men and sin: what's the difference? *Nat Rev Mol Cell Biol*, **2**, 815-826.

Barr, F.A., Puype, M., Vandekerckhove, J. and Warren, G. (1997) GRASP65, a protein involved in the stacking of Golgi cisternae. *Cell*, **91**, 253-262.

Bartholomew, C.R., Woo, S.H., Chung, Y.S., Jones, C. and Hardy, C.F. (2001) Cdc5 interacts with the Wee1 kinase in budding yeast. *Mol Cell Biol*, **21**, 4949-4959.

Beach, D., Durkacz, B. and Nurse, P. (1982) Functionally homologous cell cycle control genes in budding and fission yeast. *Nature*, **300**, 706-709.

Bellanger, J.M. and Gonczy, P. (2003) TAC-1 and ZYG-9 form a complex that promotes microtubule assembly in *C. elegans* embryos. *Curr Biol*, **13**, 1488-1498.

Benjamin, K.R., Zhang, C., Shokat, K.M. and Herskowitz, I. (2003) Control of landmark events in meiosis by the CDK Cdc28 and the meiosis-specific kinase Ime2. *Genes Dev*, **17**, 1524-1539.

Blagden, S.P. and Glover, D.M. (2003) Polar expeditions--provisioning the centrosome for mitosis. *Nat Cell Biol*, **5**, 505-511.

Blangy, A., Lane, H.A., d'Herin, P., Harper, M., Kress, M. and Nigg, E.A. (1995) Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. *Cell*, **83**, 1159-1169.

Borrelly, G.P., Harrison, M.D., Robinson, A.K., Cox, S.G., Robinson, N.J. and Whitehall, S.K. (2002) Surplus zinc is handled by Zym1 metallothionein and Zhf endoplasmic reticulum transporter in *Schizosaccharomyces pombe*. *J Biol Chem*, **277**, 30394-30400.

Branden, C. and Tooze, J. (1999) *Introduction to Protein Structure*. Garland Publishing, New York.

Bridge, A.J., Morphew, M., Bartlett, R. and Hagan, I.M. (1998) The fission yeast SPB component Cut12 links bipolar spindle formation to mitotic control. *Genes Dev*, **12**, 927-942.

Briggs, S.D., Xiao, T., Sun, Z.W., Caldwell, J.A., Shabanowitz, J., Hunt, D.F., Allis, C.D. and Strahl, B.D. (2002) Gene silencing: trans-histone regulatory pathway in chromatin. *Nature*, **418**, 498.

Browning, H., Hackney, D.D. and Nurse, P. (2003) Targeted movement of cell end factors in fission yeast. *Nat Cell Biol*, **5**, 812-818.

Budde, P.P., Kumagai, A., Dunphy, W.G. and Heald, R. (2001) Regulation of Op18 during spindle assembly in *Xenopus* egg extracts. *J Cell Biol*, **153**, 149-158.

Cambiazo, V., Logarinho, E., Pottstock, H. and Sunkel, C.E. (2000) Microtubule binding of the *Drosophila* DMAP-85 protein is regulated by phosphorylation in vitro. *FEBS Lett*, **483**, 37-42.

Carmena, M., Riparbelli, M.G., Minestrini, G., Tavares, A.M., Adams, R., Callaini, G. and Glover, D.M. (1998) *Drosophila* polo kinase is required for cytokinesis. *J Cell Biol*, **143**, 659-671.

Casenghi, M., Meraldi, P., Weinhart, U., Duncan, P.I., Korner, R. and Nigg, E.A. (2003) Polo-like kinase 1 regulates Nlp, a centrosome protein involved in microtubule nucleation. *Dev Cell*, **5**, 113-125.

Chang, F., Drubin, D. and Nurse, P. (1997) cdc12p, a protein required for cytokinesis in fission yeast, is a component of the cell division ring and interacts with profilin. *J Cell Biol*, **137**, 169-182.

Chang, F. and Nurse, P. (1996) How fission yeast fission in the middle. *Cell*, **84**, 191-194.

Chang, F., Woollard, A. and Nurse, P. (1996) Isolation and characterization of fission yeast mutants defective in the assembly and placement of the contractile actin ring. *J Cell Sci*, **109**, 131-142.

Chang, L. and Gould, K.L. (2000) Sid4p is required to localize components of the septation initiation pathway to the spindle pole body in fission yeast. *Proc Natl Acad Sci U S A*, **97**, 5249-5254.

Charles, J.F., Jaspersen, S.L., Tinker-Kulberg, R.L., Hwang, L., Szidon, A. and Morgan, D.O. (1998) The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in *S. cerevisiae*. *Curr Biol*, **8**, 497-507.

Chase, D., Serafinas, C., Ashcroft, N., Kosinski, M., Longo, D., Ferris, D.K. and Golden, A. (2000) The polo-like kinase PLK-1 is required for nuclear envelope breakdown and the completion of meiosis in *Caenorhabditis elegans*. *Genesis*, **26**, 26-41.

Chen, D., Toone, W.M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N. and Bahler, J. (2003) Global transcriptional responses of fission yeast to environmental stress. *Mol Biol Cell*, **14**, 214-229.

Cheng, L., Hunke, L. and Hardy, C.F. (1998) Cell cycle regulation of the *Saccharomyces cerevisiae* polo-like kinase cdc5p. *Mol Cell Biol*, **18**, 7360-7370.

Ciechanover, A. (1994) The ubiquitin-proteasome proteolytic pathway. *Cell*, **79**, 13-21.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M. and Nasmyth, K. (1998) An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell*, **93**, 1067-1076.

Clyne, R.K., Katis, V.L., Jessop, L., Benjamin, K.R., Herskowitz, I., Lichten, M. and Nasmyth, K. (2003) Polo-like kinase Cdc5 promotes chiasmata formation and cosegregation of sister centromeres at meiosis I. *Nat Cell Biol*, **5**, 480-485.

Cohen-Fix, O. (2001) The making and breaking of sister chromatid cohesion. *Cell*, **106**, 137-140.

Cohen-Fix, O. (2003) Meiosis: polo, FEAR and the art of dividing reductionally. *Curr Biol*, **13**, R603-605.

Compton, D.A. (1998) Focusing on spindle poles. *J Cell Sci*, **111**, 1477-1481.

Cserzo, M., Wallin, E., Simon, I., von Heijne, G. and Elofsson, A. (1997) Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. *Protein Eng*, **10**, 673-676.

Cullen, C.F., May, K.M., Hagan, I.M., Glover, D.M. and Ohkura, H. (2000) A new genetic method for isolating functionally interacting genes: high *plo1*$^{+}$-dependent mutants and their suppressors define genes in mitotic and septation pathways in fission yeast. *Genetics*, **155**, 1521-1534.

Cullen, C.F. and Ohkura, H. (2001) Msps protein is localized to acentrosomal poles to ensure bipolarity of *Drosophila* meiotic spindles. *Nat Cell Biol*, **3**, 637-642.

Davis, L. and Smith, G.R. (2001) Meiotic recombination and chromosome segregation in *Schizosaccharomyces pombe*. *Proc Natl Acad Sci U S A*, **98**, 8395-8402.

de Carcer, G., do Carmo Avides, M., Lallena, M.J., Glover, D.M. and Gonzalez, C. (2001) Requirement of Hsp90 for centrosomal function reflects its regulation of Polo kinase stability. *EMBO J*, **20**, 2878-2884.

Descombes, P. and Nigg, E.A. (1998) The polo-like kinase Plx1 is required for M phase exit and destruction of mitotic regulators in *Xenopus* egg extracts. *EMBO J*, **17**, 1328-1335.

Ding, D.Q., Tomita, Y., Yamamoto, A., Chikashige, Y., Haraguchi, T. and Hiraoka, Y. (2000) Large-scale screening of intracellular protein localization in living fission yeast cells by the use of a GFP-fusion genomic DNA library. *Genes Cells*, **5**, 169-190.

do Carmo Avides, M., Tavares, A. and Glover, D.M. (2001) Polo kinase and Asp are needed to promote the mitotic organizing activity of centrosomes. *Nat Cell Biol*, **3**, 421-424.

Donaldson, M.M., Tavares, A.A., Hagan, I.M., Nigg, E.A. and Glover, D.M. (2001) The mitotic roles of Polo-like kinase. *J Cell Sci*, **114**, 2357-2358.

Edamatsu, M. and Toyoshima, Y.Y. (1996) Isolation and characterization of pos mutants defective in correct positioning of septum in Schizosaccharomyces pombe. *Zoolog Sci*, **13**, 235-239.

Elia, A.E., Cantley, L.C. and Yaffe, M.B. (2003a) Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science*, **299**, 1228-1231.

Elia, A.E., Rellos, P., Haire, L.F., Chao, J.W., Ivins, F.J., Hoepker, K., Mohammad, D., Cantley, L.C., Smerdon, S.J. and Yaffe, M.B. (2003b) The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. *Cell*, **115**, 83-95.

Ellinger-Ziegelbauer, H., Karasuyama, H., Yamada, E., Tsujikawa, K., Todokoro, K. and Nishida, E. (2000) Ste20-like kinase (SLK), a regulatory kinase for polo-like kinase (Plk) during the G2/M transition in somatic cells. *Genes Cells*, **5**, 491-498.

Feierbach, B. and Chang, F. (2001) Cytokinesis and the contractile ring in fission yeast. *Current Opinion in Microbiology*, **4**, 713-719.

Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature*, **340**, 245-246.

Forbes, K.C., Humphrey, T. and Enoch, T. (1998) Suppressors of cdc25p overexpression identify two pathways that influence the G2/M checkpoint in fission yeast. *Genetics*, **150**, 1361-1375.

Forsburg, S.L. and Nurse, P. (1991) Cell cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Annual Review of Cell Biology*, **7**, 227-256.

Funabiki, H., Kumada, K. and Yanagida, M. (1996a) Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. *EMBO J*, **15**, 6617-6628.

Funabiki, H., Yamano, H., Kumada, K., Nagao, K., Hunt, T. and Yanagida, M. (1996b) Cut2 proteolysis required for sister-chromatid seperation in fission yeast. *Nature*, **381**, 438-441.

Geymonat, M., Jensen, S. and Johnston, L.H. (2002) Mitotic exit: the Cdc14 double cross. *Curr Biol*, **12**, R482-484.

Geymonat, M., Spanos, A., Walker, P.A., Johnston, L.H. and Sedgwick, S.G. (2003) In vitro regulation of budding yeast Bfa1/Bub2 GAP activity by Cdc5. *J Biol Chem*, **278**, 14591-14594.

Glover, D.M., Ohkura, H. and Tavares, A. (1996) Polo kinase: the choreographer of the mitotic stage? *J Cell Biol*, **135**, 1681-1684.

Golsteyn, R.M., Mundt, K.E., Fry, A.M. and Nigg, E.A. (1995) Cell cycle regulation of the activity and subcellular localization of Plk1, a human protein kinase implicated in mitotic spindle function. *J Cell Biol*, **129**, 1617-1628.

Golsteyn, R.M., Schultz, S.J., Bartek, J., Ziemiecki, A., Ried, T. and Nigg, E.A. (1994) Cell cycle analysis and chromosomal localization of human Plk1, a putative homologue of the mitotic kinases *Drosophila* polo and *Saccharomyces cerevisiae* Cdc5. *J Cell Sci*, **107**, 1509-1517.

Goode, B.L., Rodal, A.A., Barnes, G. and Drubin, D.G. (2001) Activation of the Arp2/3 complex by the actin filament binding protein Abp1p. *J Cell Biol*, **153**, 627-634.

Goto, B., Okazaki, K. and Niwa, O. (2001) Cytoplasmic microtubular system implicated in *de novo* formation of a Rabl-like orientation of chromosomes in fission yeast. *J Cell Sci*, **114**, 2427-2435.

Gould, K.L. and Simanis, V. (1997) The control of septum formation in fission yeast. *Genes Dev*, **11**, 2939-2951.

Grallert, A. and Hagan, I.M. (2002) *Schizosaccharomyces pombe* NIMA-related kinase, Fin1, regulates spindle formation and an affinity of Polo for the SPB. *EMBO J*, **21**, 3096-3107.

Gross, S.D., Schwab, M.S., Taieb, F.E., Lewellyn, A.L., Qian, Y.W. and Maller, J.L. (2000) The critical role of the MAP kinase pathway in meiosis II in *Xenopus* oocytes is mediated by $p90^{Rsk}$. *Curr Biol*, **10**, 430-438.

Gruber, S., Haering, C.H. and Nasmyth, K. (2003) Chromosomal cohesin forms a ring. *Cell*, **112**, 765-777.

Gruneberg, U. and Nigg, E.A. (2003) Regulation of cell division: stop the SIN! *Trends Cell Biol*, **13**, 159-162.

Guertin, D.A., Chang, L., Irshad, F., Gould, K.L. and McCollum, D. (2000) The role of the sid1p kinase and cdc14p in regulating the onset of cytokinesis in fission yeast. *EMBO J*, **19**, 1803-1815.

Guertin, D.A., Trautmann, S. and McCollum, D. (2002a) Cytokinesis in eukaryotes. *Microbiol Mol Biol Rev*, **66**, 155-178.

Guertin, D.A., Venkatram, S., Gould, K.L. and McCollum, D. (2002b) Dma1 prevents mitotic exit and cytokinesis by inhibiting the septation initiation network (SIN). *Dev Cell*, **3**, 779-790.

Hagan, I. and Yanagida, M. (1990) Novel potential mitotic motor protein encoded by the fission yeast *cut7*$^{+}$ gene. *Nature*, **347**, 563-566.

Hagan, I. and Yanagida, M. (1992) Kinesin-related cut7 protein associates with mitotic and meiotic spindles in fission yeast. *Nature*, **356**, 74-76.

Hall, I.M., Noma, K. and Grewal, S.I. (2003) RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. *Proc Natl Acad Sci U S A*, **100**, 193-198.

Hamanaka, R., Smith, M.R., O'Connor, P.M., Maloid, S., Mihalic, K., Spivak, J.L., Longo, D.L. and Ferris, D.K. (1995) Polo-like kinase is a cell cycle-regulated kinase activated during mitosis. *J Biol Chem*, **270**, 21086-21091.

Hannak, E., Kirkham, M., Hyman, A.A. and Oegema, K. (2001) Aurora-A kinase is required for centrosome maturation in *Caenorhabditis elegans*. *J Cell Biol*, **155**, 1109-1116.

Hardy, C.F. and Pautz, A. (1996) A novel role for Cdc5p in DNA replication. *Mol Cell Biol*, **16**, 6775-6782.

Hayles, J. and Nurse, P. (1986) Cell cycle regulation in yeast. *J Cell Sci Suppl*, **4**, 155-170.

Heald, R., Tournebize, R., Blank, T., Sandaltzopoulos, R., Becker, P., Hyman, A. and Karsenti, E. (1996) Self-organization of microtubules into bipolar spindles around artificial chromosomes in *Xenopus* egg extracts. *Nature*, **382**, 420-425.

Heald, R., Tournebize, R., Habermann, A., Karsenti, E. and Hyman, A. (1997) Spindle assembly in *Xenopus* egg extracts: respective roles of centrosomes and microtubule self-organization. *J Cell Biol*, **138**, 615-628.

Heitz, M.J., Petersen, J., Valovin, S. and Hagan, I.M. (2001) MTOC formation during mitotic exit in fission yeast. *J Cell Sci*, **114**, 4521-4532.

Honigberg, S.M. and Purnapatre, K. (2003) Signal pathway integration in the switch from the mitotic cell cycle to meiosis in yeast. *J Cell Sci*, **116**, 2137-2147.

Hoyt, M.A. (1994) Cellular roles of kinesin and related proteins. *Curr Opin Cell Biol*, **6**, 63-68.

Hu, F., Wang, Y., Liu, D., Li, Y., Qin, J. and Elledge, S.J. Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. (2001) *Cell*, **107**, 655-665.

Humphries, C.L., Balcer, H.I., D'Agostino, J.L., Winsor, B., Drubin, D.G., Barnes, G., Andrews, B.J. and Goode, B.L. (2002) Direct regulation of Arp2/3 complex activity and function by the actin binding protein coronin. *J Cell Biol*, **159**, 993-1004.

Hunter, T. and Plowman, G.D. (1997) The protein kinases of budding yeast: six score and more. *Trends Biochem Sci*, **22**, 18-22.

Jackman, M., Lindon, C., Nigg, E.A. and Pines, J. (2003) Active cyclin B1-Cdk1 first appears on centrosomes in prophase. *Nat Cell Biol*, **5**, 143-148.

Jang, Y.J., Lin, C.Y., Ma, S. and Erikson, R.L. (2002a) Functional studies on the role of the C-terminal domain of mammalian polo-like kinase. *Proc Natl Acad Sci U S A*, **99**, 1984-1989.

Jang, Y.J., Ma, S., Terada, Y. and Erikson, R.L. (2002b) Phosphorylation of threonine 210 and the role of serine 137 in the regulation of mammalian polo-like kinase. *J Biol Chem*, **277**, 44115-44120.

Jansen, R.P. (2001) mRNA localization: message on the move. *Nat Rev Mol Cell Biol*, **2**, 247-256.

Jaspersen, S.L. and Morgan, D.O. (2000) Cdc14 activates cdc15 to promote mitotic exit in budding yeast. *Curr Biol*, **10**, 615-618.

Joshi, H.C. (1994) Microtubule organizing centers and gamma-tubulin. *Curr Opin Cell Biol*, **6**, 54-62.

Kang, D., Chen, J., Wong, J. and Fang, G. (2002) The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition. *J Cell Biol*, **156**, 249-259.

Karagiannis, J., Oulton, R. and Young, P.G. (2002) The Scw1 RNA-binding domain protein regulates septation and cell-wall structure in fission yeast. *Genetics*, **162**, 45-58.

Karaiskou, A., Jessus, C., Brassac, T. and Ozon, R. (1999) Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification. *J Cell Sci*, **112**, 3747-3756.

Kelm, O., Wind, M., Lehmann, W.D. and Nigg, E.A. (2002) Cell cycle-regulated phosphorylation of the *Xenopus* polo-like kinase Plx1. *J Biol Chem*, **277**, 25247-25256.

Kilmartin, J.V. (1994) Genetic and biochemical approaches to spindle function and chromosome segregation in eukaryotic microorganisms. *Curr Opin Cell Biol*, **6**, 50-54.

Kitada, K., Johnson, A.L., Johnston, L.H. and Sugino, A. (1993) A multicopy suppressor gene of the *Saccharomyces cerevisiae* G1 cell cycle mutant gene dbf4 encodes a protein kinase and is identified as CDC5. *Mol Cell Biol*, **13**, 4445-4457.

Klein, F., Mahr, P., Galova, M., Buonomo, S.B., Michaelis, C., Nairz, K. and Nasmyth, K. (1999) A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. *Cell*, **98**, 91-103.

Koepp, D.M., Harper, J.W. and Elledge, S.J. (1999) How the cyclin became a cyclin: regulated proteolysis in the cell cycle. *Cell*, **97**, 431-434.

Kotani, S., Tugendreich, S., Fujii, M., Jorgensen, P.M., Watanabe, N., Hoog, C., Hieter, P. and Todokoro, K. (1998) PKA and MPF-activated polo-like kinase regulate anaphase-promoting complex activity and mitosis progression. *Mol Cell*, **1**, 371-380.

Kramer, J. and Hawley, R.S. (2003) The spindle-associated transmembrane protein Axs identifies a membranous structure ensheathing the meiotic spindle. *Nat Cell Biol*, **5**, 261-263.

Krapp, A., Cano, E. and Simanis, V. (2003) Mitotic hyperphosphorylation of the fission yeast SIN scaffold protein cdc11p is regulated by the protein kinase cdc7p. *Curr Biol*, **13**, 168-172.

Krapp, A., Schmidt, S., Cano, E. and Simanis, V. (2001) *S. pombe* cdc11p, together with sid4p, provides an anchor for septation initiation network proteins on the spindle pole body. *Curr Biol*, **11**, 1559-1568.

Kufer, T.A., Sillje, H.H., Korner, R., Gruss, O.J., Meraldi, P. and Nigg, E.A. (2002) Human TPX2 is required for targeting Aurora-A kinase to the spindle. *J Cell Biol*, **158**, 617-623.

Kumagai, A. and Dunphy, W.G. (1996) Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from *Xenopus* egg extracts. *Science*, **273**, 1377-1380.

Lamb, J.R., Michaud, W.A., Sikorski, R.S. and Hieter, P.A. (1994) Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis. *EMBO J*, **13**, 4321-4328.

Lamb, J.R., Tugendreich, S. and Hieter, P. (1995) Tetratrico peptide repeat interactions: to TPR or not to TPR? *Trends Biochem Sci*, **20**, 257-259.

Lane, H.A. and Nigg, E.A. (1996) Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. *J Cell Biol*, **135**, 1701-1713.

Le Goff, X., Utzig, S. and Simanis, V. (1999) Controlling septation in fission yeast: finding the middle, and timing it right. *Curr Genet*, **35**, 571-584.

Lee, B. and Amon, A. (2001) Meiosis: how to create a specialized cell cycle. *Curr Opin Cell Biol*, **13**, 770-777.

Lee, B.H. and Amon, A. (2003) Role of Polo-like kinase CDC5 in programming meiosis I chromosome segregation. *Science*, **300**, 482-486.

Lee, B.H., Amon, A. and Prinz, S. (2002) Spo13 regulates cohesin cleavage. *Genes Dev*, **16**, 1672-1681.

Lee, K.S., Grenfell, T.Z., Yarm, F.R. and Erikson, R.L. (1998) Mutation of the polo-box disrupts localization and mitotic functions of the mammalian polo kinase Plk. *Proc Natl Acad Sci U S A*, **95**, 9301-9306.

Lee, K.S., Song, S. and Erikson, R.L. (1999) The polo-box-dependent induction of ectopic septal structures by a mammalian polo kinase, plk, in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A*, **96**, 14360-14365.

Lee, K.S., Yuan, Y.L., Kuriyama, R. and Erikson, R.L. (1995) Plk is an M-phase-specific protein kinase and interacts with a kinesin-like protein, CHO1/MKLP-1. *Mol Cell Biol*, **15**, 7143-7151.

Leung, G.C., Hudson, J.W., Kozarova, A., Davidson, A., Dennis, J.W. and Sicheri, F. (2002) The Sak polo-box comprises a structural domain sufficient for mitotic subcellular localization. *Nat Struct Biol*, **9**, 719-724.

Li, C., Furge, K.A., Cheng, Q.C. and Albright, C.F. (2000) Byr4 localizes to spindle-pole bodies in a cell cycle-regulated manner to control Cdc7 localization and septation in fission yeast. *J Biol Chem*, **275**, 14381-14387.

Lin, C.Y., Madsen, M.L., Yarm, F.R., Jang, Y.J., Liu, X. and Erikson, R.L. (2000) Peripheral Golgi protein GRASP65 is a target of mitotic polo-like kinase (Plk) and Cdc2. *Proc Natl Acad Sci U S A*, **97**, 12589-12594.

Lin, H.R., Ting, N.S., Qin, J. and Lee, W.H. (2003) M phase-specific phosphorylation of BRCA2 by Polo-like kinase 1 correlates with the dissociation of the BRCA2-P/CAF complex. *J Biol Chem*, **278**, 35979-35987.

Liu, X. and Erikson, R.L. (2002) Activation of Cdc2/cyclin B and inhibition of centrosome amplification in cells depleted of Plk1 by siRNA. *Proc Natl Acad Sci U S A*, **99**, 8672-8676.

Logarinho, E. and Sunkel, C.E. (1998) The *Drosophila* POLO kinase localises to multiple compartments of the mitotic apparatus and is required for the phosphorylation of MPM2 reactive epitopes. *J Cell Sci*, **111**, 2897-2909.

Lohka, M.J., Hayes, M.K. and Maller, J.L. (1988) Purification of maturation-promoting factor, an intracelular regulator of early mitotic events. *Proc Natl Acad Sci U S A*, **85**, 3009-3013.

Lu, Y., Sugiura, R., Yada, T., Cheng, H., Sio, S.O., Shuntoh, H. and Kuno, T. (2002) Calcineurin is implicated in the regulation of the septation initiation network in fission yeast. *Genes Cells*, **7**, 1009-1019.

Lupas, A., Van Dyke, M. and Stock, J. (1991) Predicting coiled coils from protein sequences. *Science*, **252**, 1162-1164.

Lutkenhaus, J. (1993) FtsZ ring in bacterial cytokinesis. *Mol Microbiol*, **9**, 403-409.

Lutkenhaus, J. (2002) Dynamic proteins in bacteria. *Curr Opin Microbiol*, **5**, 548-552.

MacIver, F.H., Tanaka, K., Robertson, A.M. and Hagan, I.M. (2003) Physical and functional interactions between polo kinase and the spindle pole component Cut12 regulate mitotic commitment in *S. pombe*. *Genes Dev*, **17**, 1507-1523.

Marks, J., Hagan, I.M. and Hyams, J.S. (1986) Growth polarity and cytokinesis in fission yeast: the role of the cytoskeleton. *J Cell Sci Suppl*, **5**, 229-241.

Marks, J. and Hyams, J.S. (1985) Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe*. *J Cell Biol*, **39**, 27-32.

Masuda, H. (1995) The formation and functioning of yeast mitotic spindles. *Bioessays*, **17**, 45-51.

Mata, J. and Bahler, J. (2003) Correlations Between Gene Expression and Gene Conservation in Fission Yeast. *Genome Res*. **13**, 2686-2690.

Maundrell, K. (1993) Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene*, **123**, 127-130.

May, K.M., Reynolds, N., Cullen, C.F., Yanagida, M. and Ohkura, H. (2002) Polo boxes and Cut23 (Apc8) mediate an interaction between polo kinase and the anaphase-promoting complex for fission yeast mitosis. *J Cell Biol*, **156**, 23-28.

McCollum, D. and Gould, K.L. (2001) Timing is everything: regulation of mitotic exit and cytokinesis by the MEN and SIN. *Trends Cell Biol*, **11**, 89-95.

McKim, K.S., Jang, J.K. and Manheim, E.A. (2002) Meiotic recombination and chromosome segregation in *Drosophila* females. *Annu Rev Genet*, **36**, 205-232.

Meraldi, P. and Nigg, E.A. (2001) Centrosome cohesion is regulated by a balance of kinase and phosphatase activities. *J Cell Sci*, **114**, 3749-3757.

Meraldi, P. and Nigg, E.A. (2002) The centrosome cycle. *FEBS Lett*, **521**, 9-13.

Miki, F., Kurabayashi, A., Tange, Y., Okazaki, K., Shimanuki, M. and Niwa, O. (2004) Two-hybrid search for proteins that interact with Sad1 and Kms1, two membrane-bound components of the spindle pole body in fission yeast. *Mol Genet Genomics*, **270**, 449-461.

Minet, M., Nurse, P., Thuriaux, P. and Mitchison, J.M. (1979) Uncontrolled septation in a cell division cycle mutant of the fission yeast *Schizosaccharomyces pombe*. *J Bacteriol*, **137**, 440-446.

Mitchison, J.M. and Nurse, P. (1985) Growth in cell length in the fission yeast *Schizosaccharomyces pombe*. *J Cell Sci*, **75**, 357-376.

Moreno, S., Hayles, J. and Nurse, P. (1989) Regulation of $p34^{cdc2}$ protein kinase during mitosis. *Cell*, **58**, 361-372.

Morrell, J.L., Tomlin, G.C., Rajagopalan, S., Venkatram, S., Feoktistova, A.S., Tasto, J.J., Mehta, S., Jennings, J.L., Link, A., Balasubramanian, M.K. and Gould, K.L. (2004) Sid4p-Cdc11p assembles the septation initiation network and its regulators at the *S. pombe* SPB. *Curr Biol*, **14**, 579-584.

Moutinho-Santos, T., Sampaio, P., Amorim, I., Costa, M. and Sunkel, C.E. (1999) In vivo localisation of the mitotic POLO kinase shows a highly dynamic association with the mitotic apparatus during early embryogenesis in *Drosophila*. *Biol Cell*, **91**, 585-596.

Mueller, P.R., Coleman, T.R., Kumagai, A. and Dunphy, W.G. (1995) Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science*, **270**, 86-90.

Mulvihill, D.P., Barretto, C. and Hyams, J.S. (2001) Localization of Fission Yeast Type II Myosin, Myo2, to the Cytokinetic Actin Ring Is Regulated by Phosphorylation of a C-Terminal Coiled-Coil Domain and Requires a Functional Septation Initiation Network. *Mol Biol Cell*, **12**, 4044-4053.

Mulvihill, D.P. and Hyams, J.S. (2002) Cytokinetic actomyosin ring formation and septation in fission yeast are dependent on the full recruitment of the polo-like kinase Plo1 to the spindle pole body and a functional spindle assembly checkpoint. *J Cell Sci*, **115**, 3575-3586.

Mulvihill, D.P., Petersen, J., Ohkura, H., Glover, D.M. and Hagan, I.M. (1999) Plo1 kinase recruitment to the spindle pole body and its role in cell division in *Schizosaccharomyces pombe*. *Mol Biol Cell*, **10**, 2771-2785.

Murray, A.W. and Hunt, T. (1993) *The Cell Cycle: An Introduction.* Oxford University Press, Inc. New York.

Nakajima, H., Toyoshima-Morimoto, F., Taniguchi, E. and Nishida, E. (2003) Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. *J Biol Chem*, **278**, 25277-25280.

Neef, R., Preisinger, C., Sutcliffe, J., Kopajtich, R., Nigg, E.A., Mayer, T.U. and Barr, F.A. (2003) Phosphorylation of mitotic kinesin-like protein 2 by polo-like kinase 1 is required for cytokinesis. *J Cell Biol*, **162**, 863-876.

Ng, H.H., Feng, Q., Wang, H., Erdjument-Bromage, H., Tempst, P., Zhang, Y. and Struhl, K. (2002) Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. *Genes Dev*, **16**, 1518-1527.

Nigg, E.A. (1998) Polo-like kinases: positive regulators of cell division from start to finish. *Curr Opin Cell Biol*, **10**, 776-783.

Nigg, E.A., Blangy, A. and Lane, H.A. (1996) Dynamic changes in nuclear architecture during mitosis: on the role of protein phosphorylation in spindle assembly and chromosome segregation. *Exp Cell Res*, **229**, 174-180.

Niwa, O., Shimanuki, M. and Miki, F. (2001) Telomere-led bouquet formation facilitates homologous chromosome pairing and restricts ectopic interaction in fission yeast meiosis. *EMBO J*, **19**, 3831-3840.

Nurse, P., Thuriaux, P. and Nasmyth, K. (1976) Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. *Mol Gen Genet*, **146**, 167-178.

Oakley, B.R., Oakley, C.E., Yoon, Y. and Jung, M.K. (1990) Gamma-tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell*, **61**, 1289-1301.

Ohi, R. and Gould, K.L. (1999) Regulating the onset of mitosis. *Curr Opin Cell Biol*, **11**, 267-273.

Ohkura, H., Garcia, M. and Toda, T. (2001) Dis1/TOG universal microtubule adaptors - one MAP for all? *J of Cell Science*, **114**, 1-8.

Ohkura, H., Hagan, I.M. and Glover, D.M. (1995) The conserved *Schizosaccharomyces pombe* kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. *Genes Dev*, **9**, 1059-1073.

Okano-Uchida, T., Okumura, E., Iwashita, M., Yoshida, H., Tachibana, K. and Kishimoto, T. (2003) Distinct regulators for Plk1 activation in starfish meiotic and early embryonic cycles. *EMBO J*, **22**, 5633-5642.

Okazaki, K., Kurabayashi, A., Chikashige, Y., Hiraoka, Y. and Niwa, O. (2001) Possible Involvement of the SPB protein Sid4 in chromosome positioning within the nucleus. *Abstracts of the Second International Fission Yeast Meeting*, 149.

Pahlavan, G., Polanski, Z., Kalab, P., Golsteyn, R., Nigg, E.A. and Maro, B. (2000) Characterization of polo-like kinase 1 during meiotic maturation of the mouse oocyte. *Dev Biol*, **220**, 392-400.

Pardo, M. and Nurse, P. (2003) Equatorial retention of the contractile actin ring by microtubules during cytokinesis. *Science*, **300**, 1569-1574.

Park, C.J., Song, S., Lee, P.R., Shou, W., Deshaies, R.J. and Lee, K.S. (2003) Loss of CDC5 function in *Saccharomyces cerevisiae* leads to defects in Swe1p regulation and Bfa1p/Bub2p-independent cytokinesis. *Genetics*, **163**, 21-33.

Pelham, R.J. and Chang, F. (2002) Actin dynamics in the contractile ring during cytokinesis in fission yeast. *Nature*, **419**, 82-86.

Pereira, G., Manson, C., Grindlay, J. and Schiebel, E. (2002) Regulation of the Bfa1p-Bub2p complex at spindle pole bodies by the cell cycle phosphatase Cdc14p. *J Cell Biol*, **157**, 367-379.

Peters, J.M. (1994) Proteasomes: protein degradation machines of the cell. *Trends Biochem Sci*, **19**, 377-382.

Petronczki, M., Siomos, M.F. and Nasmyth, K. (2003) Un menage a quatre: the molecular biology of chromosome segregation in meiosis. *Cell*, **112**, 423-440.

Pines, J. (1993) Cyclins and cyclin-dependent kinases: take your partners. *Trends Biochem Sci*, **18**, 195-197.

Pollard, T.D. and Borisy, G.G. (2003) Cellular motility driven by assembly and disassembly of actin filaments. *Cell*, **112**, 453-465.

Qian, Y.W., Erikson, E. and Maller, J.L. (1998) Purification and cloning of a protein kinase that phosphorylates and activates the polo-like kinase Plx1. *Science*, **282**, 1701-1704.

Qian, Y.W., Erikson, E. and Maller, J.L. (1999) Mitotic effects of a constitutively active mutant of the *Xenopus* polo-like kinase Plx1. *Mol Cell Biol*, **19**, 8625-8632.

Qian, Y.W., Erikson, E., Taieb, F.E. and Maller, J.L. (2001) The polo-like kinase Plx1 is required for activation of the phosphatase Cdc25C and cyclin B-Cdc2 in Xenopus oocytes. *Mol Biol Cell*, **12**, 1791-1799.

Reynolds, N. and Ohkura, H. (2003) Polo boxes form a single functional domain that mediates interactions with multiple proteins in fission yeast polo kinase. *J Cell Sci*, **116**, 1377-1387.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. and Seraphin, B. (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol*, **17**, 1030-1032.

Riparbelli, M.G., Callaini, G. and Glover, D.M. (2000) Failure of pronuclear migration and repeated divisions of polar body nuclei associated with MTOC defects in polo eggs of *Drosophila*. *J Cell Sci*, **113**, 3341-3350.

Robinson, J.T., Wojcik, E.J., Sanders, M.A., McGrail, M. and Hays, T.S. (1999) Cytoplasmic dynein is required for the nuclear attachment and migration of centrosomes during mitosis in *Drosophila*. *J Cell Biol*, **146**, 597-608.

Roshak, A.K., Capper, E.A., Imburgia, C., Fornwald, J., Scott, G. and Marshall, L.A. (2000) The human polo-like kinase, PLK, regulates cdc2/cyclin B through phosphorylation and activation of the cdc25C phosphatase. *Cell Signal*, **12**, 405-411.

Samejima, I., Matsumoto, T., Nakaseko, Y., Beach, D. and Yanagida, M. (1993) Identification of seven new cut genes involved in *Schizosaccharomyces pombe* mitosis. *J Cell Sci*, **105**, 135-143.

Sawin, K.E. Cytokinesis: Sid signals septation. *Curr Biol*, **10**, R547-550.

Schmidt, S., Hofmann, K. and Simanis, V. (1997a) Sce3, a suppressor of the *Schizosaccharomyces pombe* septation mutant cdc11, encodes a putative RNA-binding protein. *Nucleic Acids Res*, **25**, 3433-3439.

Schmidt, S., Sohrmann, M., Hofmann, K., Woollard, A. and Simanis, V. (1997b) The Spg1p GTPase is an essential, dosage-dependent inducer of septum formation in *Schizosaccharomyces pombe*. *Genes Dev*, **11**, 1519-1534.

Sedgwick, S.G. and Smerdon, S.J. (1999) The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem Sci*, **24**, 311-316.

Shimanuki, M., Miki, F., Ding, D.Q., Chikashige, Y., Hiraoka, Y., Horio, T. and Niwa, O. (1997) A novel fission yeast gene, kms1+, is required for the formation of meiotic prophase-specific nuclear architecture. *Mol Gen Genet*, **254**, 238-249.

Shirayama, M., Matsui, Y. and Toh, E.A. (1994) The yeast TEM1 gene, which encodes a GTP-binding protein, is involved in termination of M phase. *Mol Cell Biol*, **14**, 7476-7482.

Shirayama, M., Zachariae, W., Ciosk, R. and Nasmyth, K. (1998) The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae*. *EMBO J*, **17**, 1336-1349.

Shonn, M.A., McCarroll, R. and Murray, A.W. (2002) Spo13 protects meiotic cohesin at centromeres in meiosis I. *Genes Dev*, **16**, 1659-1671.

Sikorski, R.S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19-27.

Simanis, V. (2003a) Events at the end of mitosis in the budding and fission yeasts. *J Cell Sci*, **116**, 4263-4275.

Simanis, V. (2003b) The mitotic exit and septation initiation networks. *J Cell Sci*, **116**, 4261-4262.

Sohrmann, M., Fankhauser, C., Brodbeck, C. and Simanis, V. (1996) The *dmf1/mid1* gene is essential for correct positioning of the division septum in fission yeast. *Genes Dev*, **10**, 2707-2719.

Sohrmann, M., Schmidt, S., Hagan, I. and Simanis, V. (1998) Asymmetric segregation on spindle poles of the *Schizosaccharomyces pombe* septum-inducing protein kinase Cdc7p. *Genes Dev*, **12**, 84-94.

Song, S., Grenfell, T.Z., Garfield, S., Erikson, R.L. and Lee, K.S. (2000) Essential function of the polo box of Cdc5 in subcellular localization and induction of cytokinetic structures. *Mol Cell Biol*, **20**, 286-298.

Song, S. and Lee, K.S. (2001) A novel function of *Saccharomyces cerevisiae* CDC5 in cytokinesis. *J Cell Biol*, **152**, 451-469.

Sparks, C.A., Morphew, M. and McCollum, D. (1999) Sid2p, a spindle pole body kinase that regulates the onset of cytokinesis. *J Cell Biol*, **146**, 777-790.

Stegmeier, F., Visintin, R. and Amon, A. (2002) Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. *Cell*, **108**, 207-220.

Strauss, B.S. (1999) Frameshift mutation, microsatellites and mismatch repair. *Mutat Res*, **437**, 195-203.

Sunkel, C.E. and Glover, D.M. (1988) polo, a mitotic mutant of *Drosophila* displaying abnormal spindle poles. *J Cell Sci*, **89**, 25-38.

Sutterlin, C., Lin, C.Y., Feng, Y., Ferris, D.K., Erikson, R.L. and Malhotra, V. (2001) Polo-like kinase is required for the fragmentation of pericentriolar Golgi stacks during mitosis. *Proc Natl Acad Sci U S A*, **98**, 9128-9132.

Tanaka, K., Petersen, J., MacIver, F., Mulvihill, D.P., Glover, D.M. and Hagan, I.M. (2001) The role of Plo1 kinase in mitotic commitment and septation in *Schizosaccharomyces pombe*. *EMBO J*, **20**, 1259-1270.

Tanaka, T.U., Rachidi, N., Janke, C., Pereira, G., Galova, M., Schiebel, E., Stark, M.J. and Nasmyth, K. (2002) Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. *Cell*, **108**, 317-329.

Tavares, A.A., Glover, D.M. and Sunkel, C.E. (1996) The conserved mitotic kinase polo is regulated by phosphorylation and has preferred microtubule-associated substrates in *Drosophila* embryo extracts. *EMBO J*, **15**, 4873-4883.

Tomlin, G.C., Morrell, J.L. and Gould, K.L. (2002) The spindle pole body protein Cdc11p links Sid4p to the fission yeast septation initiation network. *Mol Biol Cell*, **13**, 1203-1214.

Tong, C., Fan, H.Y., Lian, L., Li, S.W., Chen, D.Y., Schatten, H. and Sun, Q.Y. (2002) Polo-like kinase-1 is a pivotal regulator of microtubule assembly during mouse

oocyte meiotic maturation, fertilization, and early embryonic mitosis. *Biol Reprod*, **67**, 546-554.

Toyoshima-Morimoto, F., Taniguchi, E., Shinya, N., Iwamatsu, A. and Nishida, E. (2001) Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase. *Nature*, **410**, 215-220.

Trautmann, S., Wolfe, B.A., Jorgensen, P., Tyers, M., Gould, K.L. and McCollum, D. (2001) Fission yeast Clp1p phosphatase regulates G2/M transition and coordination of cytokinesis with cell cycle progression. *Curr Biol*, **11**, 931-940.

Tsvetkov, L., Xu, X., Li, J. and Stern, D.F. (2003) Polo-like kinase 1 and Chk2 interact and co-localize to centrosomes and the midbody. *J Biol Chem*, **278**, 8468-8475.

Uchiumi, T., Longo, D.L. and Ferris, D.K. (1997) Cell cycle regulation of the human polo-like kinase (PLK) promoter. *J Biol Chem*, **272**, 9166-9174.

Uetz, P., Giot, L., Cagney, G., Mansfield, T.A., Judson, R.S., Knight, J.R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., Qureshi-Emili, A., Li, Y., Godwin, B., Conover, D., Kalbfleisch, T., Vijayadamodar, G., Yang, M., Johnston, M., Fields, S. and Rothberg, J.M. (2000) A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. *Nature*, **403**, 623-627.

Uetz, P. and Hughes, R.E. (2000) Systematic and large-scale two-hybrid screens. *Curr Opin Microbiol*, **3**, 303-308.

Vale, R.D. (2003) The molecular motor toolbox for intracellular transport. *Cell*, **112**, 467-480.

Visintin, R., Stegmeier, F. and Amon, A. (2003) The role of the polo kinase cdc5 in controlling cdc14 localization. *Mol Biol Cell*, **14**, 4486-4498.

Watanabe, Y. (2003) Monopolar attachment by Polo. *Nat Cell Biol*, **5**, 379-382.

Weaver, A.M., Young, M.E., Lee, W.L. and Cooper, J.A. (2003) Integration of signals to the Arp2/3 complex. *Curr Opin Cell Biol*, **15**, 23-30.

Wianny, F., Tavares, A., Evans, M.J., Glover, D.M. and Zernicka-Goetz, M. (1998) Mouse polo-like kinase 1 associates with the acentriolar spindle poles, meiotic chromosomes and spindle midzone during oocyte maturation. *Chromosoma*, **107**, 430-439.

Yanagida, M., Yamashita, Y.M., Tatebe, H., Ishii, K., Kumada, K. and Nakaseko, Y. (1999) Control of metaphase-anaphase progression by proteolysis: cyclosome function regulated by the protein kinase A pathway, ubiquitination and localization. *Philos Trans R Soc Lond B Biol Sci*, **354**, 1559-1569; discussion 1569-1570.

Yarm, F.R. (2002) Plk phosphorylation regulates the microtubule-stabilizing protein TCTP. *Mol Cell Biol*, **22**, 6209-6221.

Yoshida, S. and Toh-e, A. (2002) Budding yeast Cdc5 phosphorylates Net1 and assists Cdc14 release from the nucleolus. *Biochem Biophys Res Commun*, **294**, 687-691.

Yuan, J., Eckerdt, F., Bereiter-Hahn, J., Kurunci-Csacsko, E., Kaufmann, M. and Strebhardt, K. (2002) Cooperative phosphorylation including the activity of polo-like kinase 1 regulates the subcellular localization of cyclin B1. *Oncogene*, **21**, 8282-8292.

Zhou, T., Aumais, J.P., Liu, X., Yu-Lee, L.Y. and Erikson, R.L. (2003) A role for Plk1 phosphorylation of NudC in cytokinesis. *Dev Cell*, **5**, 127-138.